AD_____
(Leave blank)

Award Number:

W81XWH-10-1-0365

TITLE:

Novel Therapeutic Strategy for the Prevention of Bone Fractures

PRINCIPAL INVESTIGATOR:

Mark W. Hamrick, Ph.D.

CONTRACTING ORGANIZATION:

Georgia Health Sciences University
Augusta, GA 30912

REPORT DATE:

June 2013

TYPE OF REPORT:

Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

X Approved for public release; distribution unlimited

☐ Distribution limited to U.S. Government agencies only; report contains proprietary information

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
June 2013	Final	15 May 2010-14 May 2013

4. TITLE AND SUBTITLE

Novel Therapeutic Strategy for the Prevention of Bone Fractures

5a. CONTRACT NUMBER
W81XWH-10-1-0365

5b. GRANT NUMBER
W81XWH-10-1-0365

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Mark W. Hamrick, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Georgia Health Sciences University
Augusta GA 30912

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research
& Material Command
Fort Detrick, Maryland
21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release;distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Falls and debilitating bone fractures are a major problem for veterans, and more than 40,000 veterans suffered hip fractures from 2000-2002. Falls are the main etiological factor in more than 90% of fractures, and so treatments that can improve muscle strength while at the same time increasing bone mass will significantly reduce fracture-related morbidity and mortality. Myostatin is a factor that induces muscle wasting and suppresses bone formation. Our data collected thus far demonstrate i) myostatin suppresses proliferation of aged, but not young, myoblasts, ii) myostatin is elevated with age in muscles composed primarily of slow-twitch fibers (e.g. soleus), and iii) myostatin increases muscle mass and muscle fiber size in aged mice. These findings suggest that myostatin inhibitors may have potential for suppressing muscle wasting and improving muscle repair in older individuals, but their effect on bone may be less significant.

15. SUBJECT TERMS

Aging; Osteoporosis; Fractures

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU		**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

Introduction

Loss of muscle mass with age is implicated in age-related bone loss, and muscle frailty contributes to an increased incidence of falls and fractures. Yet, the molecular mechanisms underlying age-related muscle wasting, and the ability of muscle to promote bone formation and fracture healing, are unknown. We have focused our research on the role of myostatin (GDF-8) in muscle-bone interactions in order to develop more effective treatment and prevention strategies for muscle injury, frailty, and bone fracture. We have previously shown that myostatin deficiency increases bone strength and biomineralization throughout the skeleton, and that a new myostatin inhibitor (propeptide) increases both muscle mass and bone formation (Hamrick et al., 2007, 2010; Elkasrawy and Hamrick, 2010). Our research therefore suggests that myostatin is a key factor regulating both myogenesis and osteogenesis. Although some studies have found no association between age and myostatin transcript levels in skeletal muscle (Marcell et al., 2001), others reveal a marked elevation in skeletal muscle myostatin expression with aging in humans (Leger et al., 2008). Additional research indicates that circulating levels of myostatin increase with age in men and women, and are highest in people aged 60-90 (Yarasheski et al., 2002). The latter finding suggests that myostatin is implicated in the sarcopenia of aging, hence myostatin inhibitors are likely to be useful pharmacological agents for treating age-related muscle atrophy as well as bone loss.

The goal of our CDMRP-sponsored research is to better characterize myostatin's role in age-related bone loss, so that targeted therapies to prevent bone fractures by enhancing muscle and bone strength can be developed. We hypothesize that the expression of myostatin and its receptor are elevated with aging in bone and muscle, which antagonizes the osteogenic and myogenic capacity of stem cells in these tissues, but that myostatin inhibitors will reverse this age-related decline in musculoskeletal function. Year 1 of the project was to determine how the expression of myostatin and its antagonist follistatin change with age in musculoskeletal tissues, whereas the goal of year 2 was to determine the effects of myostatin on anabolic pathways in primary bone-derived stromal cells and skeletal muscle myoblasts in vitro. Year 3 studies tested the hypothesis that a myostatin inhibitor could enhance muscle and bone mass in aged animals in vivo.

Findings to date demonstrate that myostatin is elevated in skeletal muscle of aged mice, but only in slow- but not fast-twitch muscles. Myostatin is also elevated in bone marrow from aged mice, whereas in patient samples activin A increases with age more so than myostatin. Myostatin suppressed proliferation of aged, but not young, myoblasts, and also increased the expression of differentiation markers in myoblasts from aged mice. Myostatin did not dramatically alter osteogenic differentiation of either young or aged bone marrow stromal cells; however, myostatin does have a significant inhibitory effect on proliferation of these stem cells. Our new in vivo data using a myostatin inhibitor (propeptide) show that blocking myostatin function in vivo increases muscle mass and fiber size in aged mice, but does not alter bone mass, strength, or parameters of bone formation & resorption. These data suggest that targeting myostatin may have significant therapeutic potential for improving muscle function in older adults, perhaps leading to the prevention of falls and fractures.

Body

Aim 1 (months 1-12). Determine how the expression of myostatin, its receptor, and the myostatin antagonist follistatin change with age in musculoskeletal tissues.

Task 1. Human bone marrow aspirates will be collected from relatively young (18-30) and older (50-70) patients in the MCG orthopaedic clinic. Samples from younger patients are collected as waste by-products during ACL reconstructions, whereas those from older patients are discarded during total knee and hip replacement surgery.

We investigated age-related changes in the activin A-myostatin-follistatin system using bone marrow samples from young (<50 years, n=7) and older (>70 years, n=10) knee arthroplasty

patients. Supernatant samples were analyzed using ELISA. Results indicate that follistatin and myostatin levels are not significantly altered with age in human bone marrow supernatants, whereas activin A levels increased increased by more than 120% in human bone marrow (Fig. 1). The marked increase in activin A levels with age in the patient samples was associated with a similar increase in the activin A: follistatin ratio.

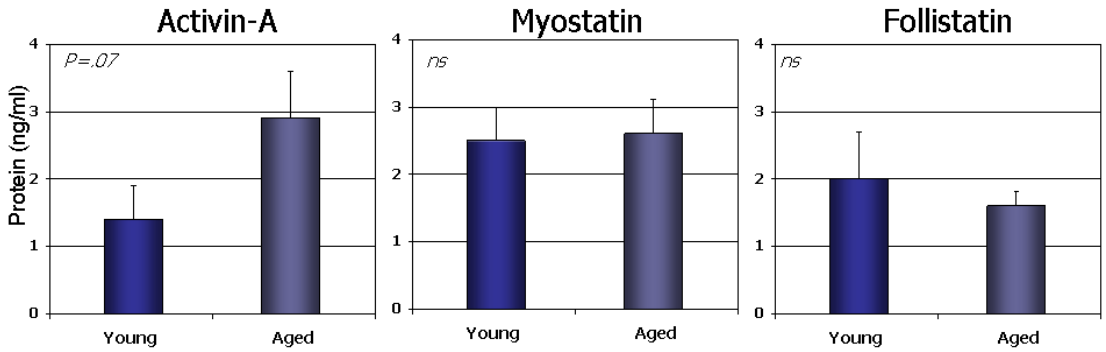


Figure 1*. Protein levels (right) of activin A, myostatin, and follistatin in bone marrow supernatants from young (<50 years) and older (>70 years) knee arthroplasty patients determined using ELISA assays.*

Task 1 status: in progress. While supernatant samples have been relatively easy to isolate, purified bone marrow stromal cells have been more challenging to retrieve from patients. We have developed a new process to isolate CD271+ cells from the bone marrow of young (under 50 yrs old) and four old (over 70 yrs old) patients using a kit (CD271 MicroBead Kit, Miltenyi Biotec Inc. CA.). We have already isolated mRNA from these cells to screen for expression of the factors show in Fig. 1 as well as the myostatin receptor, these PCR runs are being completed in June, 2013.

Task 2. Bone aspirates and muscle samples are collected from mice 12, 18 and 24 months of age, 15 mice per age group (total = 45 mice).

Protein levels of activin A, myostatin, and follistatin in skeletal muscle and bone marrow of young and aged mice were determined using ELISA. We excluded the 18 mo age group because we found in PCR assays that this group was consistently an outlier. ANOVAs performed on activin A and follistatin normalized for total protein, and the ratio of normalized activin A: follistatin, revealed no significant changes with age in either the soleus or extensor digitorum longus muscles. Levels of normalized myostatin showed a slight but non-significant decrease in the EDL with age (Fig. 2A), whereas SOL showed a significant increase in normalized myostatin with age (Fig. 2A). Likewise, the ratio of normalized myostatin: follistatin showed a significant decrease with age in EDL (Fig. 2B), but a significant increase with age in SOL (Fig. 2B). Two-factor ANOVA with age (12 or 24 mo) and muscle (EDL or SOL) as the two factors showed significant age*muscle interaction effects for both normalized myostatin ($p<.05$) and the myostatin: follistatin ratio ($p<.01$), with myostatin levels decreasing with age in the EDL but increasing with age in SOL.

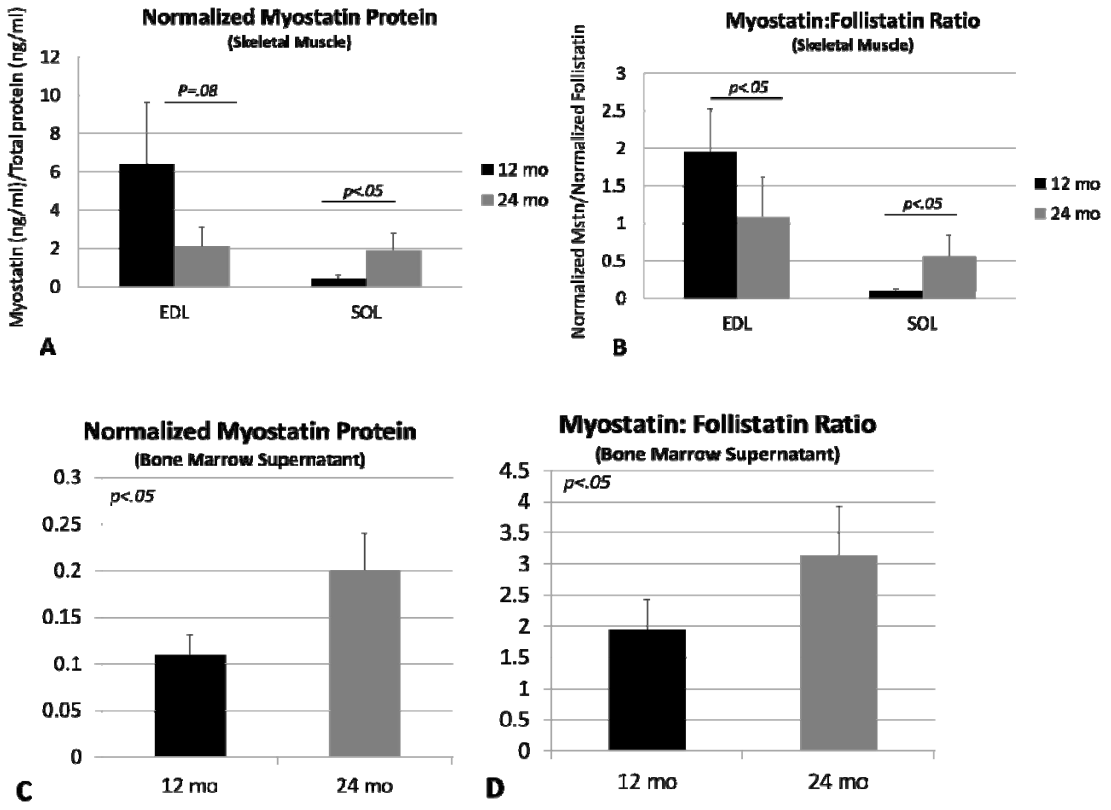


Figure 2. *Protein levels measured using ELISA of myostatin (A; normalized by BCA) and relative to follistatin (B) in the extensor digitorum longus muscle (EDL) and soleus muscle (SOL) of young (12 mo) and aged (24 mo) mice. Protein levels measured using ELISA of myostatin (C; normalized by BCA) and relative to follistatin (D) in bone marrow supernatant of young (12 mo) and aged (24 mo) mice.*

Comparisons of normalized protein levels obtained from bone marrow supernatant revealed no significant differences between older and young mice for activin A, follistatin, or the activin A: follistatin ratio. Normalized myostatin is significantly increased in mouse bone marrow with increasing age (Fig. 2C), as is the ratio of normalized myostatin: follistatin (Fig. 2D).

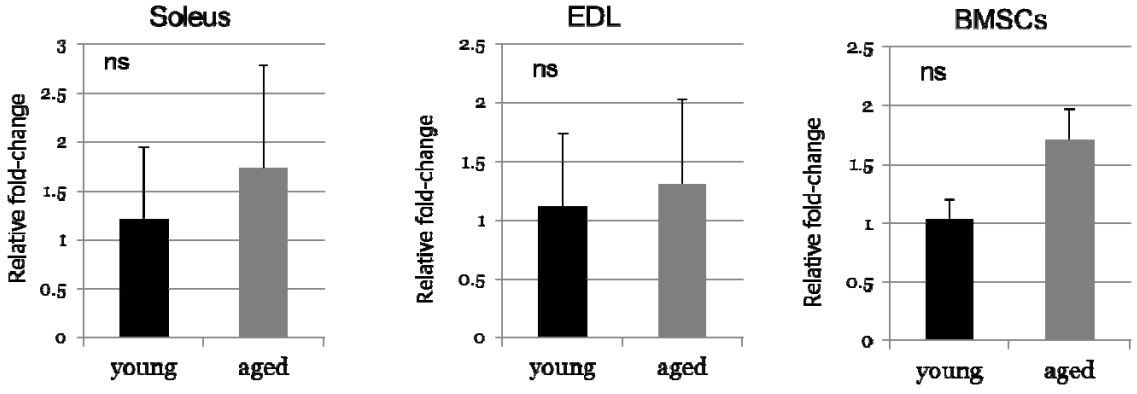


Figure 3. *Expression of the myostatin receptor (ActRIIB, or Acvr2b) in the soleus muscle (left graph), extensor digitorum longus muscle (EDL, middle graph), and in bone marrow stromal cells (BMSCs, right graph) from young (6-12 mo) and aged (24 mo) mice. Expression shows a slight but non-significant increase with age in muscle and bone tissues.*

Task 2 status. Complete.

Milestone 1: Age-related changes in the expression of myostatin, its receptor, and the myostatin antagonist follistatin in muscle and bone tissues will be defined. Although some assays on patient bone marrow stem cells are in progress, milestone 1 has been reached, with the conclusion that myostatin levels in bone marrow and slow twitch muscle fibers increase slightly with age; however, expression of the myostatin receptor does not increase significantly with age in these same tissues. Activin A levels also increase with age in human bone marrow, but as shown below this does not have a detrimental effect on bone marrow stem cells and may actually enhance their proliferation and inhibit their differentiation toward fat cells.

Aim 2 (months 12-24). Determine the effects of myostatin on anabolic pathways in primary bone-derived stromal cells and skeletal muscle myoblasts in vitro.

Task 1 (months 12-24). Human bone marrow stromal cells from young and aged patients have been collected during year 1 for specific aim 1. In year 2 these cells will be treated with myostatin, and myostatin inhibitors, in dose-response studies. We have completed proliferation assays on these cells, are currently performing the osteogenic and adipogenic differentiation assays.

Task 1 status: in progress. As noted above, purified bone marrow stromal cells were more challenging to retrieve and culture from patients. We have developed a new process to isolate CD271+ cells from the bone marrow of young (under 50 yrs old) and four old (over 70 yrs old) patients using a kit (CD271 MicroBead Kit, Miltenyi Biotec Inc. CA.). We have already isolated mRNA from these cells to screen for expression of the factors show in Fig. 1 as well as the myostatin receptor. These experiments will be completed by September 1, 2013.

Task 2. Primary myoblasts and BMSCs from young and aged mice will have been collected during year 1 for specific aim 1. In year two these cells will be treated with myostatin, and myostatin inhibitors, in dose-response studies.

Primary myoblast experiments for aim 2, task 2: For culture and treatment of primary myoblasts, tibialis anterior muscles were dissected and placed in sterile PBS. The muscle was minced with a sterile scalpel under aseptic conditions. Minced muscle was digested in 0.2% collagenase type II (Gibco) for 1 hour with frequent shaking followed by digestion in 1x trypsin for 30 minutes. The slurry was pelletted and trypsin supernatant removed. The slurry was re-suspended in proliferation medium. Upon completion of enzymatic digest, slurry was poured over a 70μm cell strainer (Fisher) to remove any remaining connective tissue. The cells were then added to collagen type I (BD Bioscience) coated T-25 flasks. Primary myoblasts were allowed to attach for 72 hours. Cells were then maintained in proliferation medium (PM): DMEM (Hyclone) supplemented with 10% fetal bovine serum, 10% horse serum, 1% penicillin / streptomycin, and 0.5% chick embryo extract (Sera Labs U.K.). Medium was changed every 48 hours until T-25 flask was confluent. Once confluent, cells were trypsinized and counted using NucleoCounter (New Brunswick Scientific). Cells were then plated in a 96 well plate at 5,000 cells/ cm2. Cells were allowed to attach in proliferation medium for 48 hours. Proliferation medium was removed, cells washed with PBS , and DMEM supplemented with 1% I.T.S was added followed by either control (PBS) or high or low dose activin A (50 ng/ml, 100 ng/ml), follistatin (100 ng/ml, 1000 ng/ml) or myostatin (100 ng/ml, 1000 ng/ml) (R&D Systems, Minneapolis). Doses follow those utilized by He et al. (2005) for activin A and Zhu et al. (2007) for myostatin and follistatin. After 24 hours of treatment, MTT reagent was added according to manufacturer's protocol and O.D. was determined 2 hours later.

For differentiation assays, cells were isolated and cultured for one week until confluent as described above. Cells were then trypsinized and plated in 12 well plates at 5,000 cells/ml and allowed to attach overnight in proliferation medium. PM was removed, cells washed with PBS. DMEM supplemented with 1% I.T.S followed by the addition of Mstn, Fstn, Activin A or control.

Cells were maintained in treatment for 48 hour then harvested in TRIZOL® reagent (Invitrogen) for RNA isolation and subsequent cDNA synthesis (Bio-Rad). 50-100 ng of cDNA was amplified in duplicates in each 40-cycle reaction using an iCycler™ (Bio-Rad) with annealing temperature set at 60ºC, ABsolute™ QPCR SYBR® Green Fluorescein Mix (ABgene, Thermo Fisher Scientific), and custom-designed qRT-PCR primers (Table 1). A melt curve was used to assess the purity of amplification products. mRNA levels were normalized to β-Actin/18S and gene expression was calculated as fold change using the comparative CT method. If not otherwise indicated, treated groups were compared to PBS control groups.

ANOVAs showed significant treatment effects for activin A, myostatin, and follistatin treatment of primary myoblasts (Fig. 4A). Activin A produced a significant dose-response increase in proliferation in myoblasts from younger mice but not older mice; however, the treatment*age interaction was not significant. Follistatin also increased proliferation in dose-response manner in young myoblasts, but the effect was attenuated in myoblasts from older mice (Fig. 4B). There was a significant (p=.001) treatment*age interaction effect for follistatin treatment where the treatment effect was much greater in younger myoblasts compared to older cells. Myostatin treatment also increased proliferation in young myoblasts compared to untreated cells, whereas in older myoblasts myostatin decreased proliferation at the low dose (Fig. 4C). There was also a significant (p<.001) treatment*age interaction effect for myostatin, where myostatin treatment increased proliferation in young myoblasts but decreased proliferation in the older cells.

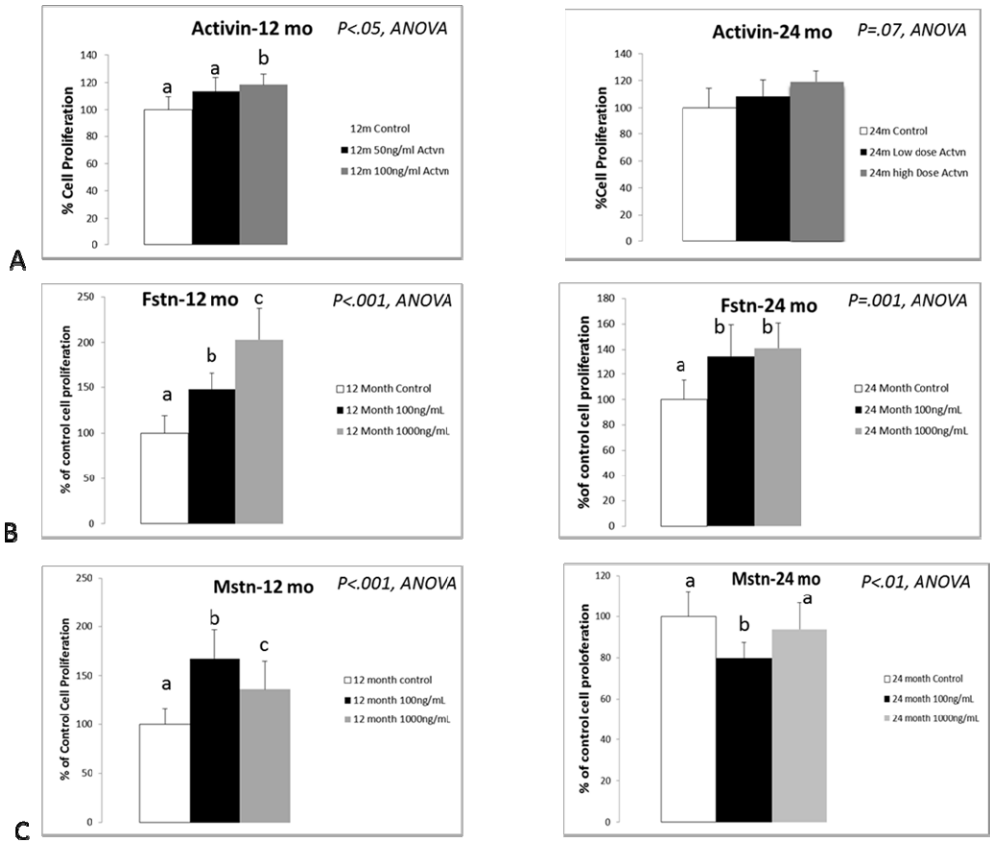


Figure 4. *Proliferation assays of primary myoblasts isolated from young (12 mo, left column) and aged (24 mo, right column) mice treated with activin A (A), follistatin (B), or myostatin (C).*

RT-PCR data revealed no marked changes in the expression of differentiation markers MyoD, myogenin, or myosin heavy chain (MHC) with Activin A treatment. MyoD and MHC expression were also unaffected by follistatin treatment of young and aged myoblasts; however, follistatin stimulated a significant increase in myogenin expression in aged but not young myoblasts (Fig. 5A). ANOVAs demonstrated a significant age effect for myogenin expression (p<.05), and there was also a significant treatment * age interaction for myogenin expression. Myostatin treatment

had no effect on MyoD or myogenin expression in either young or aged myoblasts, but myostatin did produce a significant increase in MHC expression in aged but not young myoblasts (Fig. 5B).

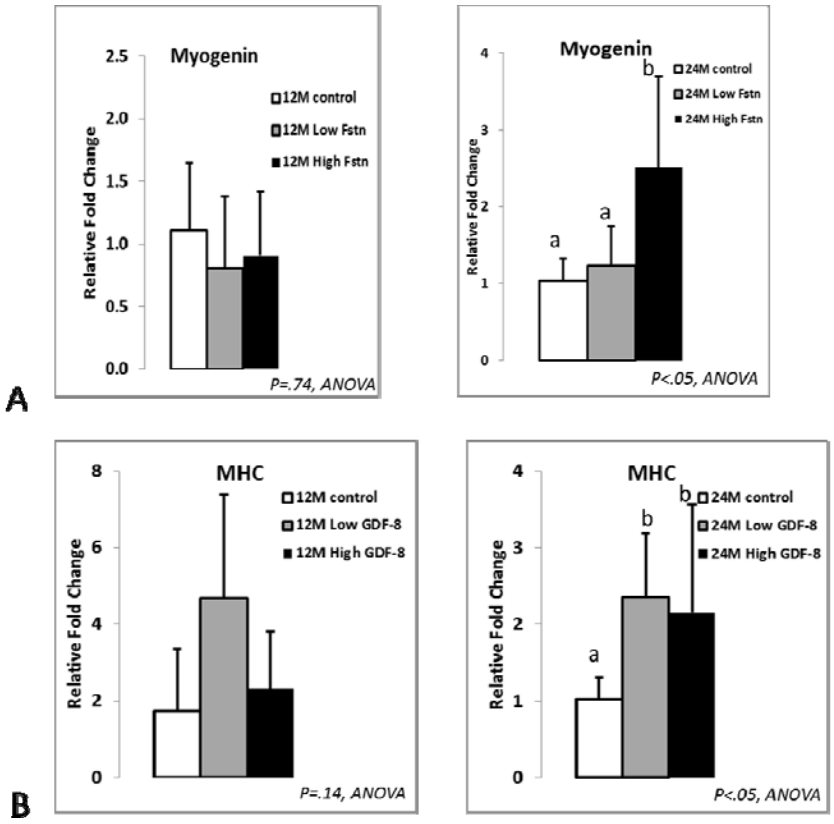


Figure 5*. Gene expression of the differentiation marker myogenin (top) and myosin heavy chain (MHC) in young (12M, left column) and aged (24M, right column) primary myoblasts treated with follistatin (A) and myostatin (B; GDF-8).*

Primary BMSC experiments for Aim 2, task 2: In Aim 1 we found that activin A was also elevated with age in bone marrow, and so we extended our treatments in aim 2 to include activin A, myostatin, and follistatin. Bone marrow aspirates were flushed from femora and magnetic nanoparticles conjugated to anti-mouse CD11b, CD45R/B220, and Pan DC monoclonal antibodies were used to remove hematopoietic-lineage cells. A round of positive-selection was then performed using anti-Sca-1 microbeads. Enriched BMSCs were cultured in proliferation medium (DMEM supplemented with 10% heat-inactivated FBS) in T-75 flasks until ~80% confluent. Cells were then lifted with trypsin/EDTA, plated in 96-well plates at a density of 5,000 cells/well in proliferation medium, and allowed to attach for 24 h. Proliferation medium was removed, cells washed with PBS, and DMEM supplemented with with 2% heat-inactivated FBS (for BMSCs) was added followed by control (PBS), activin A, follistatin, or myostatin (all from R&D Systems, Minneapolis) at the same doses noted above for primary myoblasts. After 24 h of treatment, MTS reagent was added according to the manufacturer's protocol (Promega, Madison, WI) and absorbance at 492 nm was read 2 h later. Osteogenic differentiation and Alizarin Red S (ARS) staining was performed as described previously (Zhang et al., 2008)

ANOVAs showed a significant (P<.001) age effect in each treatment group, with younger BMSCs overall having higher values for proliferation than older BMSCs, irrespective of the treatment (Fig. 6). Proliferation assays showed no effects of activin treatment on either young or old BMSCs (Fig. 6A). Two-factor ANOVA revealed a significant treatment*age interaction for follistatin, with follistatin having no impact on proliferation in older BMSCs but reducing

proliferation in young BMSCs (Fig. 6B). Myostatin significantly decreased BMSC proliferation in both age groups (Fig. 6C); however, this effect was greater in the aged cells, and this treatment*age interaction was also significant (P<.05) for myostatin.

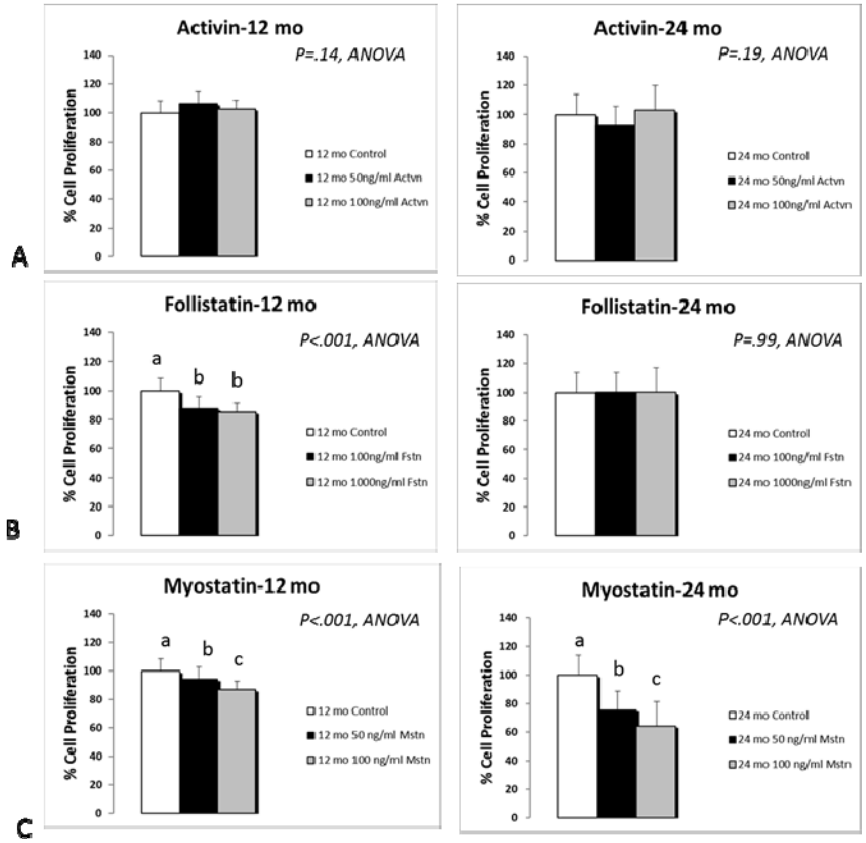


Figure 6*. Results of proliferation assays following treatment of primary bone marrow stromal cells (BMSCs) with activin A (A; activin), follistatin (B; Fstn), and myostatin (C; Mstn). Means with different superscripts differ significantly from one another (P<.05).*

Differentiation assays using alizarin red staining to detect mineralization revealed that activin treatment significantly increased mineralization of young and older BMSCs (Fig. 7). The effect of age on mineralization was significant (P<.001) for activin, with the younger cells consistently showing greater mineralization in response to treatment. Treatment effects were less pronounced in aged BMSCs, with the low dose of activin increasing alizarin red staining but the other doses showing no significant effect (Fig. 7C, D). The treatment*age interaction was significant (P<.01) for activin using two-factor ANOVA. In the myostatin experiments, the higher dose of myostatin significantly decreased mineralization in younger cells but not in older cells, and the treatment*age interaction was also significant for myostatin (P<.01). Finally, follistatin treatment did not significantly affect mineralization either young or older BMSCs (Fig. 7).

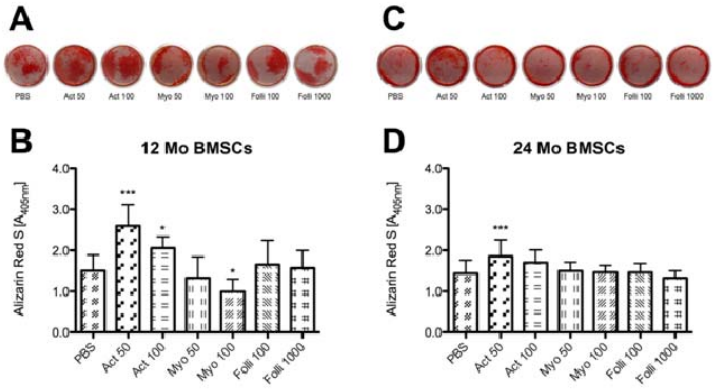


Figure 7*. Alizarin red staining of bone marrow stromal cells cultured in osteogenic conditions. Images of wells (top row) and quantification of staining (bottom row) in BMSCs from young mice (A, B; 12 Mo BMSCs) and older mice (C, D; 24 Mo BMSCs) treated with Activin A (Act), Myostatin (Myo) or Follistatin (Folli) at 50 ng/ml (50), 100 ng/ml (100) or 1000 ng/ml (1000). ***P<.001, *P<.05 relative to same-aged PBS controls.*

Task 2 status: Completed. There are two myostatin inhibitors that can be used, one that is myostatin-specific (propeptide) and one that binds both myostatin and activin A (decoy receptor). Prior to determining which is optimal we needed, in aim 2, to determine the effects of myostatin and activin on muscle and bone cells. Our data indicate that activin A can have positive effects on myoblast proliferation, and activin also enhances mineralization. Thus, a decoy receptor is not an optimal inhibitor because of its effect of blocking activin activity. While we still need to complete treatments of human BMSCs, it is clear that a myostatin-specific inhibitor (propeptide) is likely the optimal therapeutic to be validated in aim 3.

Milestone 2: The optimal treatment and dose of myostatin inhibitor for enhancing osteogenesis and myogenesis in aged BMSCs and myoblasts will have been determined in vitro. Our data in aim 2 indicate that the myostatin propeptide is the optimal inhibitor because of the contrasting effects of myostatin vs activin A in vitro.

Aim 3 (months 24-36). Determine the effects of myostatin inhibitors on muscle and bone aging in vivo.

Task 1 (months 24-30). Mice 22 months of age will be treated with a myostatin propeptide. Effects on behavioral performance and measures of bone and muscle anabolism will be determined.

Animals & treatments: C57BL6 mice were purchased from the aged rodent colony at the National Institute on Aging, National Institutes of Health (USA) at 22 months of age and delivered to Georgia Regents University, Augusta GA. Animals were allowed to acclimate for one week and were maintained at the Laboratory Animal Service Facility of Georgia Regents University. An earlier dose-response study was used to evaluate the efficacy of a myostatin propeptide in vivo (Hamrick et al., 2010). Adult mice (5-6 mo.) were treated with the propeptide at 0, 10, 20, or 50 mg/kg at day 0, 5, and 10 and then sacrificed one week after the last treatment. Those data showed that propeptide treatment increased fore- and hindlimb muscle mass by 10% at the 10 mg/kg dose and increased muscle mass by more than 15% at the 20 mg/kg dose, but the 50 mg/kg dose did not increase muscle mass beyond the increase observed in the 20 mg/kg group (Hamrick et al., 2010). The 20 mg/kg dose was therefore used in this study. Mice were divided into two treatment groups: a vehicle group (VEH; n=14) and a myostatin propeptide group (PRO; n=15). Mice received i.p. injections every five days for 25 days with a dosage of 20 mg/kg body weight at a volume of 0.2 ml. We used a four- rather than eight-week treatment period because the animals were old enough we were concerned they might die of natural causes prior to completion of the study. Myostatin propeptide [4.48mg/ml] was obtained from Pfizer Inc (Cambridge, MA, USA). Mice were given calcein i.p. injections to label actively mineralizing bone surfaces four days and 24 hours prior to sacrifice.

Myostatin propeptide increases muscle mass and fiber size in aged mice: Body weight of the vehicle- and propeptide-treated animals was similar at the end of the study (Fig 8A). Each treatment group did, however, lose some weight over the treatment period but this was less dramatic for the treated animals, such that their decrease in body weight from day 0 to day 25 was significantly less than that of the vehicle-treated mice (Fig. 8B).

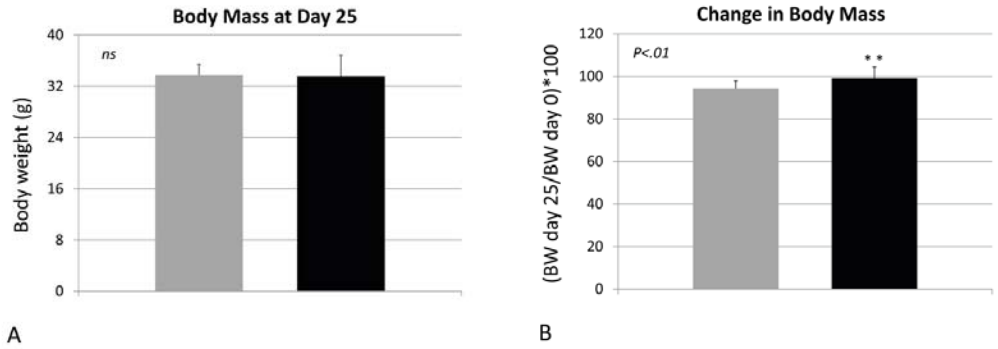


Figure 8*. Body mass (A) and change in body weight (B) for animals treated with either saline (VEH) or myostatin propeptide (PRO) weekly for over four weeks.*

Muscle mass of the tibialis anterior was significantly increased in the treated mice, both absolutely (Fig. 9A) and relative to body weight (Fig. 9B). Fiber size of the predominantly fast-twitch extensor digitorum longus (EDL) muscle was also significantly increased by more than 15% in the treated mice (Fig. 9C), whereas the increase in muscle fiber size in the predominantly slow-twitch soleus (SOL) muscle was also increased significantly (P<.05) but by a lesser magnitude (~5%).

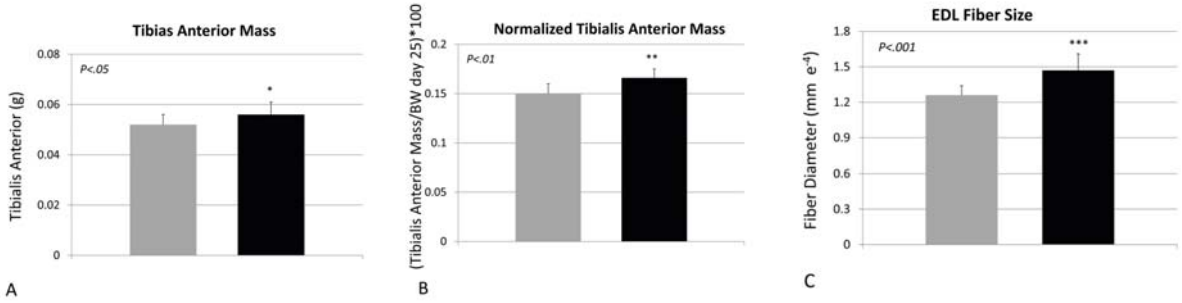


Figure 9*. Muscle parameters for mice treated with saline (VEH) or myostatin propeptide (PRO) weekly for a period of four weeks. (A) Tibialis anterior mass, (B) tibialis anterior mass relative to body weight, (C) extensor digitorum longus fiber diameter (EDL).*

Propeptide treatment produced a slight but non-significant increase in the expression of myostatin itself, as well as expression of myosin heavy chain and IGF-1 (Fig. 10). Surprisingly, expression of the ubiquitin ligases Murf1 and Mafbx was significantly increased with propeptide treatment (Fig. 10), and the PCR data were further validated by Western blot (Fig. 10).

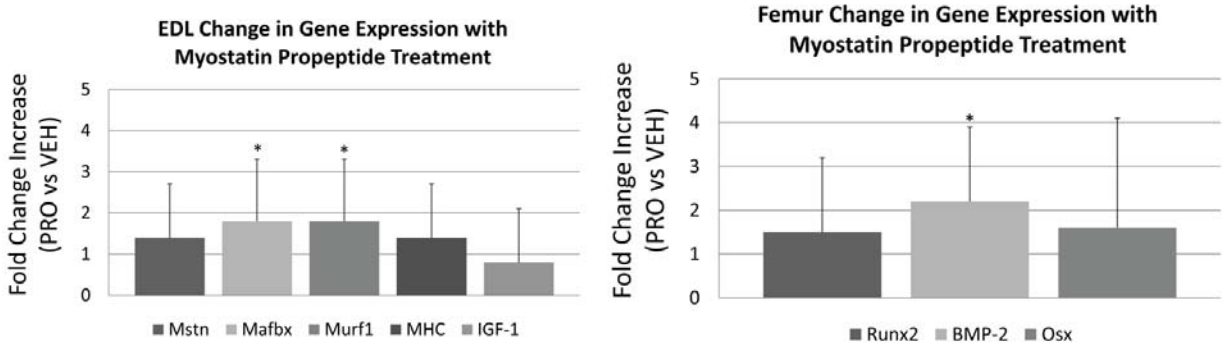


Figure 10*. Real-time PCR data for mice treated with saline (VEH) or myostatin propeptide (PRO) weekly for a period of four weeks showing increased expression of Murf1 and Mafbx in PRO-treated mice (left), and increased expression of BMP-2 in mice treated with propeptide (right). *P<.05.*

Myostatin inhibitor does not alter bone formation or bone strength in aged mice: MicroCT data from the tibia show that bone mineral density is actually slightly higher (3%) in the tibias of

vehicle-treated mice (Table 1), but other parameters such as bone volume relative to total volume, trabecular number, and trabecular thickness are similar between the two groups (Table 1). Likewise, three-point bending tests of tibias show that ultimate force, stiffness, and toughness (energy to fracture) are also similar between the vehicle- and propeptide-treated mice (Table 1).

***Table 1**. microCT and biomechanical testing of the proximal tibia for mice treated with saline (VEH) or myostatin propeptide (PRO; 20 mg/kg). BMD=bone mineral density, BV/TV=bone volume relative to total volume, Tb.Th=trabecular thickness, Tb.N=trabecular number, Fu=ultimate force, U=energy-to-fracture, S=stiffness.*

Parameter	VEH (n=14)	PRO (n=15)	p value
BMD	1.43±0.06	1.38±0.05	.01
BV/TV	6.67±2.37	6.14±2.16	.24
Tb. Th	0.11±0.02	0.11±0.01	.47
Tb. N	0.59±0.14	0.54±0.16	.23
Fu (kg)	2.21±.40	2.18±.34	.39
U (kg/um^2)	740.6±417.5	670.3±309	.31
S (g/um)	4.6±2.0	4.7±2.0	.44

Bone histomorphometry data reveal that osteoblast and osteoclast numbers do not differ between the experimental groups (Table 2). Fluorochrome labeling showed double-labels in only three mice from each group, and so single-labeled surfaces were compared. Actively mineralizing surfaces were also similar between the two groups of mice (Table 2).

***Table 2**. Bone histomorphometry data for the distal femur of mice treated with saline (VEH) or myostatin propeptide (PRO; 20 mg/kg).N.Ob/BS=osteoblast number per bone surface, MS/BS=mineralizing surface (single-label) relative to bone surface, N.Oc/BS=osteoclast number per bone surface.*

Parameter	VEH (n=15)	PRO (n=14)	p value
N.Ob/BS	27.26±17.49	25.09±9.31	.14
MS/BS	0.41±0.17	0.43±0.13	.34
N.Oc/BS	6.33±2.61	6.18±3.82	.38

Gene expression data show no significant differences in the expression of osteogenic genes Osx or Runx2 with propeptide treatment, however the expression of BMP-2 is increased in animals receiving the propeptide (Fig. 10).

Our data show that PRO treatment significantly increases muscle fiber size and muscle mass, both absolutely and relative to body weight. In contrast bone volume, bone strength, and histomorphometric parameters of bone formation and bone resorption were unchanged with PRO treatment. Our findings are consistent with previous studies utilizing a myostatin antibody in aged mice showing that targeting myostatin increases muscle fiber size and mass; however, our data differ from work recently published by Chiu et al. (2013, *J. Gerontol. A Biol. Sci. Med. Sci*) utilizing a decoy myostatin receptor (ActRIIB-Fc) showing that ActRIIB-Fc appears particularly effective at increasing bone density and bone formation. The anabolic effects of ActRIIB-Fc on aged bone are likely due to the ability of this molecule to antagonize other ligands besides myostatin, such as activin or bone morphogenetic proteins. There are several additional points to consider though when comparing the effects of the two inhibitors. If increases in muscle mass with inhibitor treatment might prevent or delay, not necessarily reverse, bone loss then a myostatin propeptide or antibody could be a safe and effective prophylactic approach for age-related bone loss. Perhaps more importantly, as noted below, the decoy receptor does not appear to be safe.

Task 1 status: The overall goals of task 1 were completed; however, we had a pathogen outbreak in our animal colony and were unable to move the mice to the behavioral core facility for rotarod and grip strength testing. We have requested a no-cost extension for FY14 to complete these behavioral studies.

Task 2 (months 30-36). Mice 22 months of age will be treated with a soluble decoy myostatin receptor for 8 weeks. Effects on behavioral performance and measures of bone and muscle anabolism will be determined.

Task 2 status. Since the CDMRP application was submitted, Acceleron decided to cease their Phase II trial of the soluble decoy myostatin receptor (ACE-03) because of safety issues (http://quest.mda.org/news/ace-031-clinical-trials-duchenne-md-stopped-now). Acceleron then announced that they will no longer pursue ACE-031 (http://www.acceleronpharma.com/2013/05/acceleron-and-shire-conclude-collaboration-on-ace-031/) this drug because of the safety issues. We therefore decided not to pursue task 2, since the molecule now appears to be unsafe in humans, and because our data in aim 2 indicate that activin A can have positive effects on myoblast proliferation, and activin also enhances mineralization. Thus, a decoy receptor is not an optimal inhibitor because of its effect of blocking activin activity

Milestone 3: The optimal myostatin inhibitor for enhancing osteogenesis and myogenesis in aged rodents will be determined in vivo. The data presented in aim 3, when taken in consideration of the Acceleron trials noted above, suggest that a myostatin propeptide is likely to be a safe and effective molecule for enhancing muscle mass with aging.

Key Research Accomplishments in Year 3:

- We published (*Experimental Gerontology*) the first data comparing the effects of myostatin treatment on young vs aged muscle and progenitor cells.
- Presented an international webinar through the International Bone & Mineral Society featuring these results.
- Completed the first in vivo studies using a myostatin propeptide in aged animals, showing for the first time that this molecule can increase muscle mass in aged, senescent muscle. This paper is in press at *Experimental Gerontology*.

Reportable Outcomes:

Manuscripts:

in press Arounleut P, Bialek P, Liang L, Upadhyay S, Fulzele S, Johnson M, Elsalanty M, Isales CM, **Hamrick MW**. A Myostatin Inhibitor (Propeptide-Fc) Increases Muscle Mass and Muscle Fiber Size in Aged Mice but Does not Increase Bone Density or Bone Strength. *Experimental Gerontology*.

2013 **Hamrick MW**, Bowser M, Chutkan N, Martell J, Corpe S, Park MA, Hillman D, Ahsan S, Arounleut P, Isales CM, Shi XM. Age-related changes in the activin A-myostatin-follistatin system within the bone marrow microenvironment. *Experimental Gerontology* 48: 290-97.

2013 Elkasrawy M, **Hamrick MW**. Myostatin (GDF-8) signaling in progenitor cells and applications to bone repair. In *Stem Cells & Bone Tissue* (R. Rajendram, V. Preedy, V. Patel, eds.), Ch. 8, pp. 145-160. CRC Press: London.

2012 **Hamrick MW**. The skeletal muscle secretome: an emerging player in muscle-bone crosstalk. *Nature Bonekey* 1: 60.

2012 Elkasrawy M, Fulzele S, Bowser M, Wenger K, **Hamrick MW**. Myostatin (GDF-8) inhibits chondrogenesis and chondrocyte proliferation in vitro by suppressing Sox-9 expression. *Growth Factors* 29:253-262.

2011 **Hamrick, MW**. A role for myokines in muscle-bone interactions. *Exercise & Sports Science Reviews* 39: 43-47.

Abstracts from Professional Presentations:

2012 Shi X, Bowser M, Yang N, He L, Herberg S, Fulzele S, Hill WD, Isales CM, Hamrick MW. Effects of Activin A and Follistatin on the Differentiation of Aged Primary Bone Marrow Stromal Cells (BMSCs) and Primary Myoblasts in vitro. J Bone Miner Res Supplement: SU 0179.

2012 Bowser M, Fulzele S, Ahsan S, Arounleut P, Isales CM, **Hamrick MW**. Changes in the activin A-myostatin-follistatin system within bone and muscle of aging mice. FASEB J 26: 914.4.

2012 Elkasrawy M, Fulzele S, Hill W, Isales CM, **Hamrick MW**. Myostatin (GDF-8) suppresses Wnt/β-catenin signaling during chondrogenesis in vitro. The Hilton Head Workshop on Regenerative Medicine.

2011 Bowser M, Chutkan N, Martell J, Corpe R, Isales CM, Park MA, Hillman D, **Hamrick MW**. Age-related changes in the activin A-myostatin-follistatin system within the bone marrow microenvironment. Journal of Bone & Mineral Research SA0001.

2011 Zhang W, **Hamrick MW**, Ding K, Wenger K, Hill W, Isales CM, Shi XM. Bone marrow mesenchymal stem cell and bone loss with aging. ASBMR Forum on Aging & Skeletal Health 29: P8.

2011 Isales CM, **Hamrick MW**, Ding K, Zhong Q, Bollag W, Shi XM, Hill W, Rowse J, Elsalanty M, Chutkan N, Insogna K. The impact of dietary protein on bone mass and strength in the aging animal. ASBMR Forum on Aging & Skeletal Health 34: P17.

2011 Elkasrawy M, Immel D, Wen X, Liu L, Lian L, **Hamrick MW**. Effects of myostatin on muscle and bone healing following deep penetrant musculoskeletal injury. British Journal of Bone & Joint Science: P053.

Invited Seminars:

2013 Progress in Bone Biology: Ageing & the Skeleton, Bone Academy, Vienna, Austria.

2013 International Webinar, International Bone & Mineral Society, New Directions in Muscle-Bone Interactions.

2012 The Hilton Head Workshop on Regenerative Medicine, Hilton Head, SC.

2012 Symposium on Muscle-Bone Crosstalk, American Physiological Society, Experimental Biology, San Diego, California.

2011 Plenary Symposium on Muscle-Bone Interactions, American Society for Bone & Mineral Research, San Diego, California.

2011 Augusta Research Symposium on Advances in Warrior Care, Augusta, GA.

2011 Program in Musculoskeletal Research, Eli Lilly & Co., Indianapolis, Indiana.

2011 Department of Pathology & Anatomical Sciences, University of Missouri, Columbia MO.

Conclusions:
Falls and debilitating bone fractures are a major problem for veterans, and more than 40,000 veterans suffered hip fractures from 2000-2002. Men have a higher fracture-related mortality than women, and one out of every three male veterans that sustains a hip fracture dies within one year. Falls are the main etiological factor in more than 90% of fractures, and so treatments that can improve muscle strength while at the same time increasing bone mass will significantly reduce fracture-related morbidity and mortality. Myostatin is a factor that induces muscle wasting and suppresses bone formation. Our data collected thus far demonstrate i) myostatin suppresses proliferation in aged, but not young, myoblasts, ii) myostatin is elevated with age in muscles composed primarily of slow-twitch fibers (e.g. soleus), and iii) myostatin increases muscle mass and muscle fiber size in aged mice. These findings suggest that myostatin inhibitors may have potential for suppressing muscle wasting and improving muscle repair in older individuals, but their effect on bone may be less significant.

References:

Chiu, C.S., Peekhaus, N., Weber, H., Adamski, S., Murray, E.M., Zhang, H.Z., Zhao, J.Z., Ernst, R., Lineberger, J., Huang, L., Hampton, R., Arnold, B.A., Vitelli, S., Hamuro, L., Wang, W.R., Wei, N., Dillon, G.M., Miao, J., Alves, S.E., Glantschnig, H., Wang, F., Wilkinson, H.A. 2013. Increased Muscle Force Production and Bone Mineral Density in ActRIIB-Fc-Treated Mature Rodents. J. *Gerontol. A Biol. Sci. Med. Sci*., Mar 22. [Epub ahead of print]

Elkasrawy M, Hamrick MW. 2010. Myostatin (GDF-8) as a key factor linking muscle mass and bone structure. *Journal of Musculoskeletal and Neuronal Interactions* 10: 56-63.

Hamrick MW, Arounleut P, Kellum E, Cain M, Immel D, Liang L. 2010. Recombinant myostatin (GDF-8) propeptide enhances the repair and regeneration of both muscle and bone in a model of deep penetrant musculoskeletal injury. *Journal of Trauma* 69: 579-83.

Hamrick MW, Shi X, Zhang W, Pennington C, Kang B, Thakore H, Haque M, Isales CM, S. Fulzele, K. Wenger. 2007. Loss of myostatin function increases osteogenic differentiation of bone marrow-derived mesenchymal stem cells but the osteogenic effect is ablated with unloading. *Bone* 40: 1544-1553.

He, L., Vichev, K., Macharia, R., Huang, R., Christ, B., Patel, K., Amthor, H. (2005). Activin A inhibits formation of skeletal muscle during chick development. Anat. Embryol. 209, 401-7.

Leger B, Derave W, De Bock K, Hespel P, Russell AP (2008) Human sarcopenia reveals an increase in SOCS-3 and myostatin and a reduced efficiency of Akt phosphorylation. Rejuvenation Res 11: 163-175B.

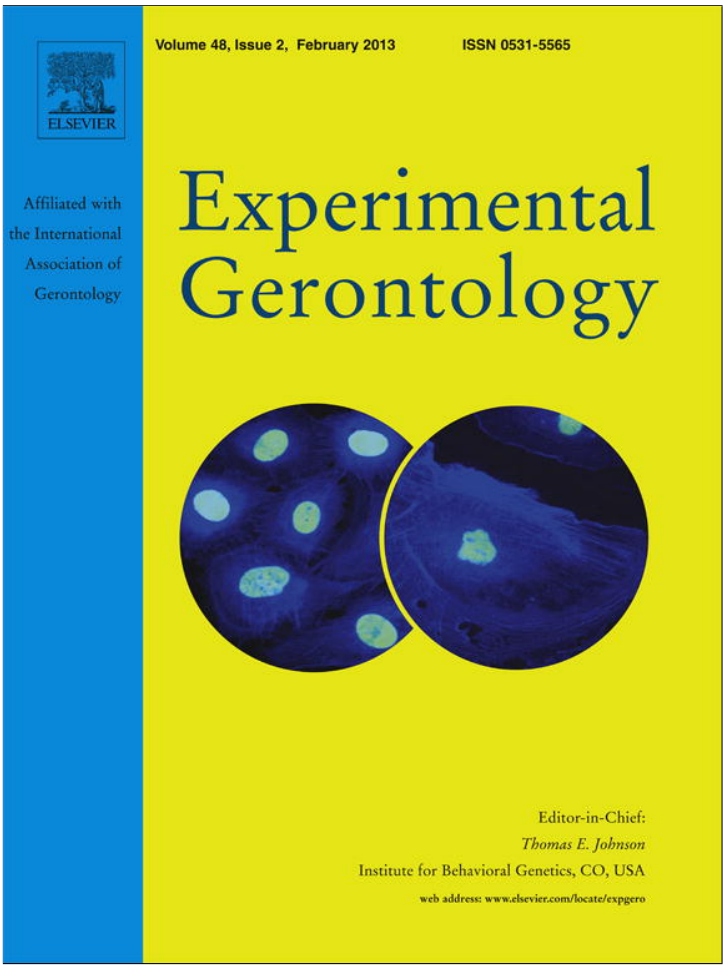
Marcell TJ, Harman SM, Urban RJ, Metz DD, Rodgers BD, Blackman MR (2001) Comparison of GH, IGF-1, and testosterone with mRNA of receptors and myostatin in older men. Am J phys Endocrinol Metab 281: E1159-64.

Yarasheski KE, Ghasin S, Sinha-Hikim I, Pak-Loduca J, Gonzalez-Cadavid NF (2002) Serum myostatin-immunoreactive protein is increased in 60-92 year old women and men with muscle wasting. J Nutr Health Aging 6: 343-8.

Zhang,W., Ou,G., Hamrick,M., Hill,W., Borke,J., Wenger,K., Chutkan,N., Yu,J., Mi,Q.S., Isales, C.M. et al. (2008). Age-Related Changes in the Osteogenic Differentiation Potential of Mouse Bone Marrow Stromal Cells. J. Bone Miner. Res. 23, 1118-1128.

Zhu, J., Li, Y., Shen, W., Qiao, C., Ambrosio, F., Lavasani, M., Nozaki, M., Branca, M., Huard, J., (2007). Relationships between transforming growth factor-β1, myostatin, and decorin. J. Biol. Chem. 282, 25852-25863.

Appendices: Articles in review, in press, and published.



Experimental Gerontology 48 (2013) 290–297

Contents lists available at SciVerse ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Effects of the activin A–myostatin–follistatin system on aging bone and muscle progenitor cells

Matthew Bowser, Samuel Herberg, Phonepasong Arounleut, Xingming Shi, Sadanand Fulzele, William D. Hill, Carlos M. Isales, Mark W. Hamrick *

Georgia Health Sciences University, Augusta, GA 30912, USA

ARTICLE INFO

Article history:
Received 25 September 2012
Received in revised form 8 November 2012
Accepted 11 November 2012
Available online 21 November 2012

Section Editor: P.J. Hornsby

Keywords:
Sarcopenia
Myoblasts
Bone marrow stromal cells
Proliferation
Differentiation

ABSTRACT

The activin A–myostatin–follistatin system is thought to play an important role in the regulation of muscle and bone mass throughout growth, development, and aging; however, the effects of these ligands on progenitor cell proliferation and differentiation in muscle and bone are not well understood. In addition, age-associated changes in the relative expression of these factors in musculoskeletal tissues have not been described. We therefore examined changes in protein levels of activin A, follistatin, and myostatin (GDF-8) in both muscle and bone with age in C57BL6 mice using ELISA. We then investigated the effects of activin A, myostatin and follistatin on the proliferation and differentiation of primary myoblasts and mouse bone marrow stromal cells (BMSCs) in vitro. Myostatin levels and the myostatin:follistatin ratio increased with age in the primarily slow-twitch mouse soleus muscle, whereas the pattern was reversed with age in the fast-twitch extensor digitorum longus muscle. Myostatin levels and the myostatin:follistatin ratio increased significantly (+75%) in mouse bone marrow with age, as did activin A levels (+17%). Follistatin increased the proliferation of primary myoblasts from both young and aged mice, whereas myostatin increased proliferation of younger myoblasts but decreased proliferation of older myoblasts. Myostatin reduced proliferation of both young and aged BMSCs in a dose-dependent fashion, and activin A increased mineralization in both young and aged BMSCs. Together these data suggest that aging in mice is accompanied by changes in the expression of activin A and myostatin, as well as changes in the response of bone and muscle progenitor cells to these factors. Myostatin appears to play a particularly important role in the impaired proliferative capacity of muscle and bone progenitor cells from aged mice.

1. Introduction

Aging is associated with a number of changes in the musculoskeletal system, including progressive deterioration of articular cartilage in the form of osteoarthritis, loss of muscle mass in the form of sarcopenia, and loss of bone density and strength in the form of osteoporosis. Muscle weakness and frailty contribute directly to postural instability, which in turn increases the risk for falls, and falls are the main etiological factor in more than 90% of bone fractures. The more than 1.5 million osteoporotic fractures a year in the US place significant burden on the healthcare system, and also contribute to significant morbidity and poor quality of life. Treatments that can improve muscle strength and at the same time increase bone mass will therefore significantly reduce fracture-related morbidity and mortality.

The activin A–myostatin–follistatin system is believed to play an important role in musculoskeletal growth, development and aging. Myostatin (GFD-8) and activin A bind type II activin receptors and signal through a transforming growth factor-beta signaling pathway involving SMAD phosphorylation. Activin is thought to bind with greater affinity to the type IIA activin receptor (ActRIIA) and myostatin to the type II B receptor (ActRIIB), but both are involved in the regulation of muscle mass (Gilson et al., 2009; Lee et al., 2010). Follistatin antagonizes both myostatin and activin A activity, and mice overexpressing follistatin in skeletal muscle show a more dramatic phenotype than mice lacking myostatin alone (Lee et al., 2005). These data suggest that alterations in either myostatin or activin A with aging or disuse can have significant effects on muscle mass, and these may be further influenced by relative levels of follistatin. Although myostatin is not highly expressed by bone cells, loss of myostatin function is associated with increased bone density in mice (Elkasrawy and Hamrick, 2010; Morissette et al., 2009). The increased bone density of mice lacking myostatin is likely multifactorial, and may result not only from the indirect effects of increased muscle mass (Hamrick, 2011, 2012) but also from increased circulating levels of IGF-1 (Williams et al., 2011). The type IIA and type IIB activin receptors are both expressed by chondrocytes and osteoblasts, and activin A has been observed to inhibit mineralization by osteoblasts in vitro whereas follistatin can increase mineralization (Eijken et al., 2007). Furthermore, inhibiting activin A in

* Corresponding author at: Department of Cellular Biology & Anatomy, Laney Walker Blvd. CB2915, Georgia Health Sciences University, Augusta, GA 30912, USA. Tel.: +1 706 721 1958; fax: +1 706 721 6120.
E-mail address: mhamrick@georgiahealth.edu (M.W. Hamrick).

0531-5565/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.exger.2012.11.004

vivo using a decoy soluble activin A receptor (ActRIIA) increases bone formation in mice (Pearsall et al., 2008).

The activin A–myostatin–follistatin system therefore appears to play a number of important roles in muscle as well as in bone metabolism. Although some studies have found no association between age and myostatin transcript levels in skeletal muscle (Marcell et al., 2001), others reveal a marked elevation in skeletal muscle myostatin expression with aging in humans (Leger et al., 2008). Additional research suggests that circulating levels of myostatin increase with age in men and women, and are highest in people aged 60–90 (Yarasheski et al., 2002). The latter finding may implicate myostatin in the sarcopenia of aging, hence myostatin inhibitors could be useful pharmacological agents for treating age-related muscle atrophy as well as bone loss. Indeed, a myostatin inhibitor has been shown to improve muscle regeneration in aged mice (Siriett et al., 2007), and a recent study reveals that muscle-derived stem cells from older male patients show a +65% higher level of myostatin expression compared to stem cells from younger patients (McKay et al., 2012). Myostatin levels can also be reduced in skeletal muscle with resistance exercise in older men (Dalbo et al., 2011), and aerobic exercise can decrease myostatin expression in older women (Konopka et al., 2010). While there has been considerable interest in the role of myostatin in musculoskeletal changes with aging, much less is known about activin A and follistatin. Circulating activin A levels have been observed to increase with age, whereas circulating follistatin levels did not show age-associated changes (Baccarelli et al., 2001; Hurwitz and Santoro, 2004). Very little information exists, however, with regard to tissue-specific changes in activin A and follistatin levels with aging or how the response of muscle and bone cells to these factors is altered with age.

We have previously shown that aged C57BL/6 mice share a number of key features in common with the aging human musculoskeletal system including an age-related decline muscle mass, both absolutely and relative to body mass, as well as loss of bone density, bone formation, and bone strength (Hamrick et al., 2006). In order to address several of the questions outlined above we examined age-related changes in the expression of activin A, follistatin, and myostatin in mouse skeletal muscle as well as in mouse bone marrow supernatant. We also investigated the response of primary muscle and bone cells from young (12 months) and aged (24 months) mice to activin A, follistatin, and myostatin treatment in vitro.

2. Materials and methods

2.1. Animals

C57BL6 mice were purchased from aged rodent colony at National Institute on Aging, National Institutes of Health (USA) at 12 and 24 months of age and delivered to Georgia Health Sciences University, Augusta, GA. Animals were allowed to acclimate for approximately two weeks and were maintained at the Laboratory Animal Service Facility of the Georgia Health Sciences University. Animals were sacrificed by CO_2 overdose and thoracotomy following procedures approved by the Institutional Animal Care and Use Committee of Georgia Health Sciences University.

Table 1
List of oligonucleotide primer sequences for qRT-PCR.

Name	Sequence	Amplicon size
Myogenin	Fwd: GGAAGTCTGTGTCGGTGGAC Rev: CGCTGCGCAGGATCTCCAC	150
MyoD	Fwd: GCCTGAGCAAAGTGAATGAG Rev: GGTCCAGGTGCGTAGAAGG	184
Myosin, heavy polypeptide 3 (Myh3)	Fwd: ACAGTCAGAGGTGTGACTCAGCCG Rev: CCGACTTGCGGAGGAAAGGTGC	90
BMP-2	Fwd: TGTTTGGCCTGAAGCAGAGA Rev: TGAGTGCCTGCGGTACAGAT	83
Osteocalcin	Fwd: ATTTAGGACCTGTGCTGCCCTA Rev: GGAGCTGCTGTGACATCCATAC	120

2.2. ELISA assays

Whole extensor digitorum longus or soleus muscle was dissected from C57BL6 mice at either 12 or 24 months of age and snap-frozen in liquid nitrogen. Each muscle was placed in 1 ml phosphate buffered saline (PBS) and subjected to homogenization using Fisherbrand Tissuemiser® rotary homogenizer until large pieces of muscle were no longer visible. Samples were subjected to two freeze–thaw cycles to disrupt the plasma membrane then centrifuged briefly. Samples were separated into 250 µl aliquots and stored at −80 °C until assayed. Follistatin and Activin A ELISA kits were purchased from R&D Systems and assays performed according to manufacturer's protocol without sample dilution. Myostatin ELISA kits were purchased from Alpco diagnostic and also performed according to manufacturer's protocol, but samples for myostatin assay were diluted 5-fold. Total protein was measured for each sample using Pierce BCA Protein Assay Kit (Thermo Scientific) according to manufacturer's protocol. Briefly: after thawing samples for ELISA, 25 µl of sample was incubated undiluted with 200 µl of Pierce® working reagent for 20 min at 37 °C. Absorbance was recorded at 590 nm wavelength and total protein concentrations were calculated based on standard curve generated with bovine serum albumin.

Left and right femora were dissected from C57BL6 mice at either 12 or 24 months of age and placed in PBS. Samples were immediately minced using fine-point surgical scissors. Samples were vortexed vigorously and triturated with a pipette to liberate marrow from bone. Samples were centrifuged briefly, supernatant was collected, and bone fragments were discarded. Supernatant was centrifuged at

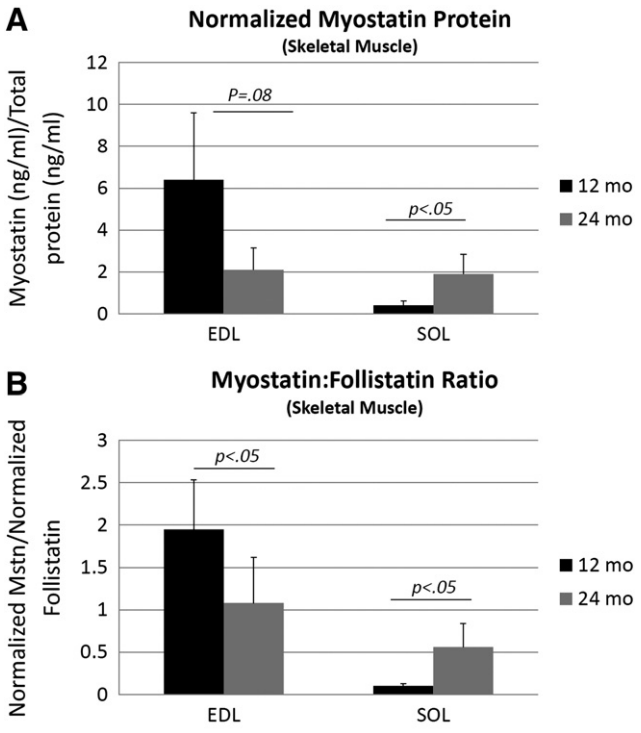


Fig. 1. Myostatin protein normalized to total protein (A) and the ratio of normalized myostatin to follistatin (B) in the extensor digitorum longus muscle (EDL) and soleus (SOL) of mice 12 months of age (12 mo) and 24 months of age (24 mo). Myostatin levels decline with age in the EDL but increase with age in the soleus. Error bars represent one standard deviation and sample size includes six replicates per group.

1500 *g* for 5 min to pellet cells. Supernatant was collected, aliquoted, and stored at −80 °C until assayed. Specific ELISA assays and total protein were performed and measured as described above for skeletal muscle homogenates.

2.3. Primary myoblast isolation, culture, and proliferation and differentiation assays

Mice 12 and 24 months of age were euthanized and tibialis anterior muscle dissected and place in sterile PBS. The muscle was minced with a sterile scalpel under aseptic conditions. Minced muscle was digested in 0.2% collagenase type II (Gibco) for 1 h with frequent shaking followed by digestion in 1× trypsin for 30 min. The slurry was pelletted and trypsin supernatant removed. The slurry was re-suspended in proliferation medium. Upon completion of enzymatic digest, slurry was poured over a 70 μm cell strainer (Fisher) to remove any remaining connective tissue. The cells were then added to collagen type I (BD Bioscience) coated T-25 flasks.

Primary myoblasts were allowed to attach for 72 h. Cells were then maintained in proliferation medium (PM): DMEM (Hyclone) supplemented with 10% fetal bovine serum, 10% horse serum, 1% penicillin/streptomycin, and 0.5% chick embryo extract (Sera Labs U.K.). Medium was changed every 48 h until T-25 flask was confluent. Once confluent, cells were trypsinized and counted using NucleoCounter (New Brunswick Scientific). Cells were then plated in a 96 well plate at 5000 cells/cm^2. Cells were allowed to attach in proliferation medium for 48 h. Proliferation medium was removed, cells washed with PBS, and DMEM supplemented with 1% insulin–transferrin–sodium selenite (ITS) was added followed by either control (PBS) or high or low dose activin A (50 ng/ml and 100 ng/ml), follistatin (100 ng/ml and 1000 ng/ml) or myostatin (100 ng/ml and 1000 ng/ml) (R&D Systems, Minneapolis). Doses follow those utilized by He et al. (2005) for activin A and Zhu et al. (2007) for myostatin and follistatin. After 24 h of treatment, MTS reagent was added according to the manufacturer's protocol (Promega, Madison) and absorbance at 492 nm was read 2 h later.

For differentiation assays, cells were isolated and cultured for one week until confluent as described above. Cells were then trypsinized and plated in 12 well plates at 5000 cells/ml and allowed to attach overnight in proliferation medium. PM was removed, cells washed with PBS, and DMEM-supplemented with 1% ITS was added followed by the addition of Mstn, Fstn, Activin A or control. Cells were

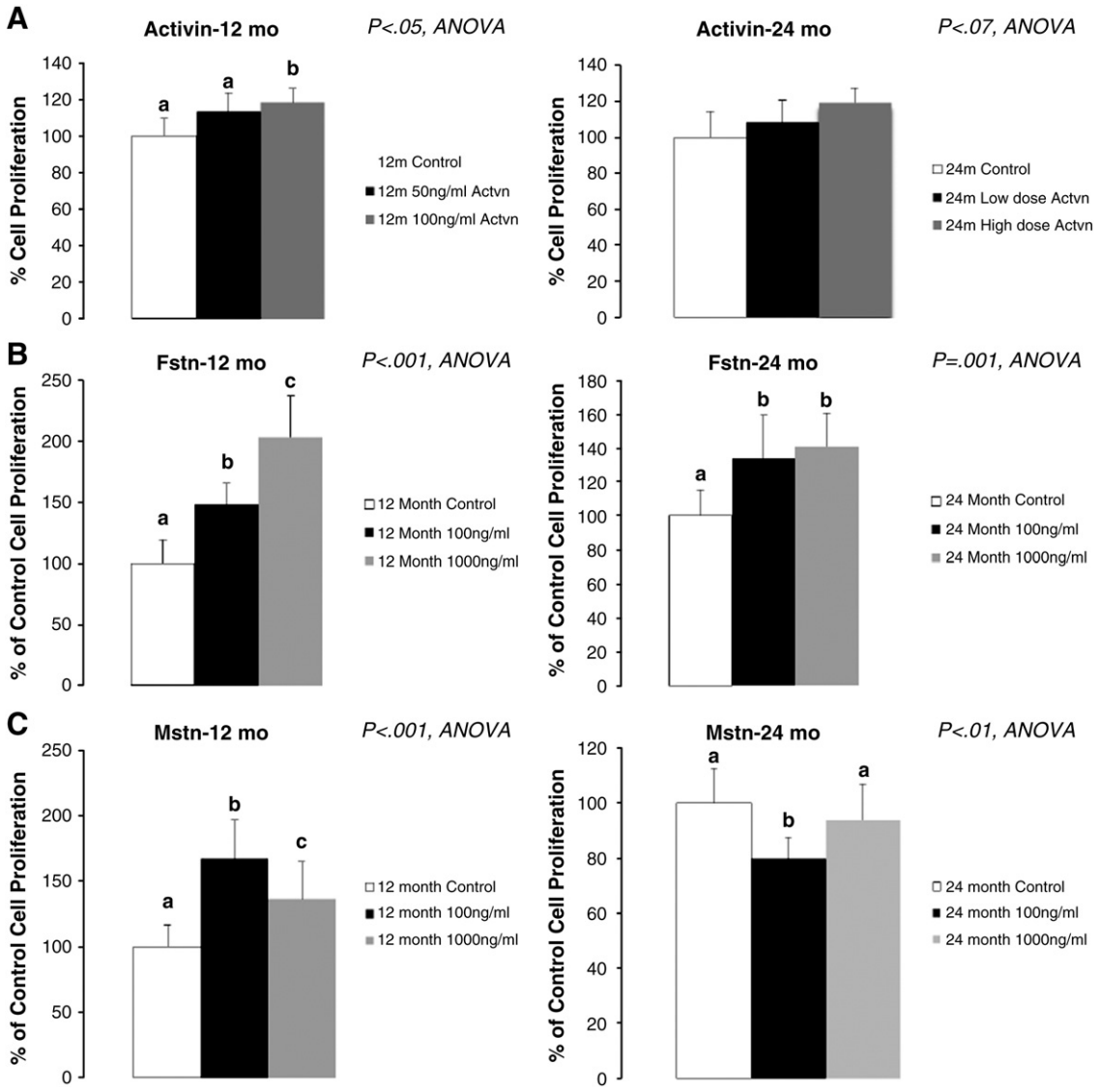


Fig. 2. Results of proliferation assays following treatment of primary myoblasts with activin A (A; activin), follistatin (B; Fstn), and myostatin (C; Mstn). Means with different superscripts differ significantly from one another (P<.05). Error bars represent one standard deviation and sample size includes eight replicates per group.

maintained in treatment for 48 h then harvested in TRIZOL® reagent (Invitrogen) for RNA isolation and subsequent cDNA synthesis (Bio-Rad). 50–100 ng of cDNA was amplified in duplicates in each 40-cycle reaction using an iCycler™ (Bio-Rad) with annealing temperature set at 60 °C, ABsolute™ QPCR SYBR® Green Fluorescein Mix (ABgene, Thermo Fisher Scientific), and custom-designed qRT-PCR primers (Table 1). A melt curve was used to assess the purity of amplification products. mRNA levels were normalized to β-Actin/18S and gene expression was calculated as fold change using the comparative CT method. If not otherwise indicated, treated groups were compared to PBS control groups.

2.4. Primary bone marrow stromal cell (BMSC) isolation, culture, and proliferation and differentiation assays

Bone marrow aspirates were flushed from mouse femora and tibias and BMSCs isolated using magnetic bead sorting as previously described (Zhang et al., 2008). Briefly, magnetic nanoparticles conjugated to anti-mouse CD11b, CD11c, CD45R/B220, and Pan DC monoclonal antibodies were used to remove hematopoietic-lineage cells and those that were negative for these four antigens remained in the solution and were collected and subjected to a round of positive-selection using anti-Sca-1 microbeads.

Enriched BMSCs were cultured in proliferation medium (DMEM supplemented with 10% heat-inactivated FBS) in T-75 flasks until ~80% confluent. Cells were then lifted with trypsin/EDTA, plated in 96-well plates at a density of 5000 cells/well in proliferation medium, and allowed to attach for 24 h. Proliferation medium was removed, cells washed with PBS, and DMEM supplemented with 2% heat-inactivated FBS (for BMSCs) was added followed by control (PBS), activin A, follistatin, or myostatin (all from R&D Systems, Minneapolis) at the same doses noted above for primary myoblasts. After 24 h of treatment, MTS reagent was added according to the manufacturer's protocol (Promega, Madison, WI) and absorbance at 492 nm was read 2 h later.

Osteogenic differentiation and Alizarin Red S (ARS) staining was performed as described previously (Zhang et al., 2008). In brief, enriched BMSCs were plated in 24-well plates at a density of 5000 cells/cm^2 in proliferation medium and allowed to attach for 24 h. Cells were then treated with osteogenic induction medium (DMEM) supplemented with 5% FBS, 0.25 mM ascorbic acid, 0.1 μM dexamethasone, and 10 mM β-glycerophosphate (all from Sigma-Aldrich) for 14 d. For ARS staining, differentiated BMSCs were washed with PBS and fixed in 3% paraformaldehyde (Sigma-Aldrich) for 30 min. Cells were stained with 40 mM ARS pH 4.1 (Sigma-Aldrich) for 15 min followed by washing with excess dH_2O. Stained plates were scanned for visualization. For quantitative destaining, 10% acetic acid was added for 30 min. Samples were transferred to a 1.5 ml microcentrifuge tube, overlaid with mineral oil (Sigma-Aldrich), heated to 85 °C for 10 min, and transferred to ice for 5 min. Following centrifugation at 14,000 rpm for 15 min, supernatants were removed and neutralized with 10% ammonium hydroxide. Aliquots were transferred to a 96-well plate and absorbance was read at 405 nm (Gregory et al., 2004). Expression of osteogenic

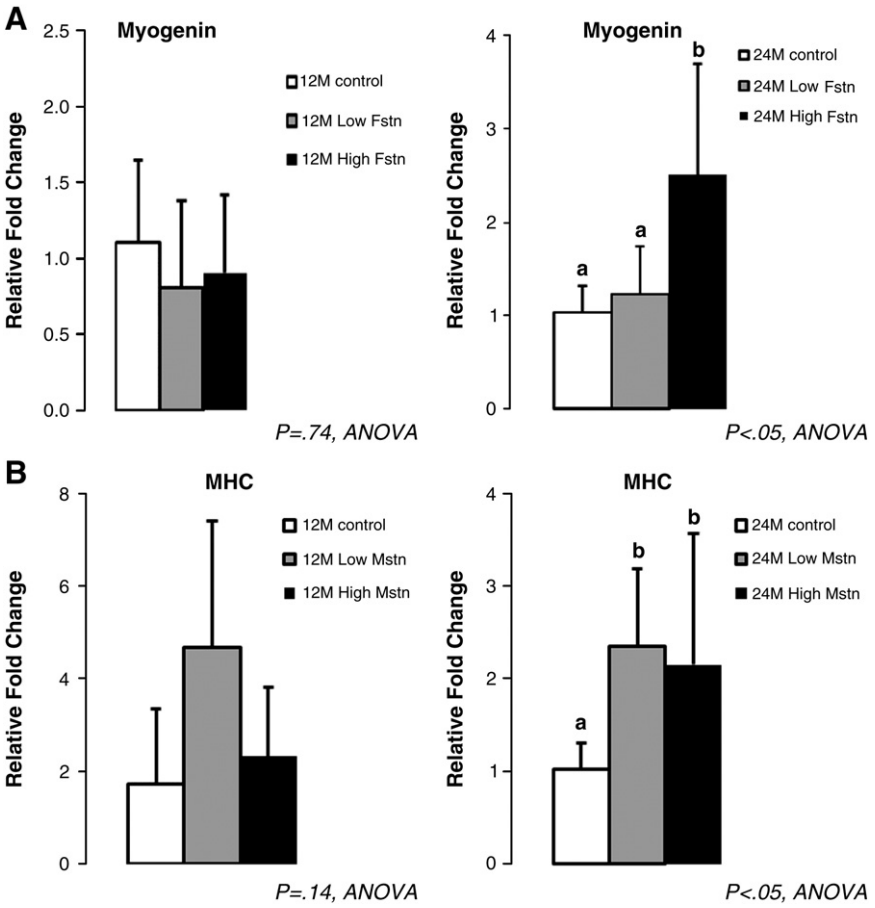


Fig. 3. Real-time PCR data for primary myoblast expression of myogenin (A) and myosin heavy chain (B) in response to treatment with follistatin (Fstn, top) and myostatin (Mstn, bottom). Means with different superscripts differ significantly from one another (P<.05). Error bars represent one standard deviation and sample size includes four-six replicates per group.

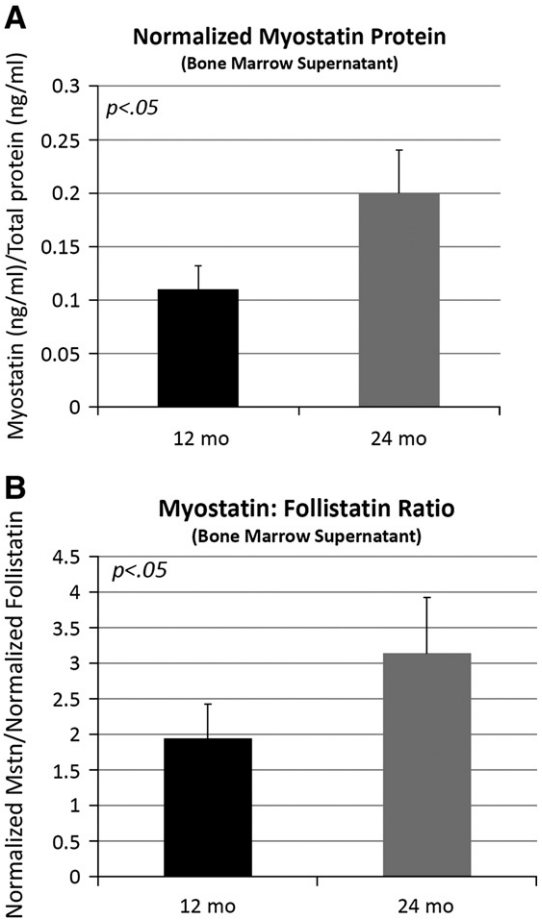


Fig. 4. Myostatin protein normalized to total protein (A) and the ratio of normalized myostatin to follistatin (B) in bone marrow supernatants from mice 12 months of age (12 mo) and 24 months of age (24 mo). Error bars represent one standard deviation and sample size includes six replicates per group.

genes was assessed using primers for bone morphogenetic protein-2 (BMP-2) and osteocalcin (OCN) shown in Table 1.

2.5. Statistical analysis

Two-factor ANOVAs were performed with treatment and age as the two factors. Fisher's least significant difference (LSD) test was used for post-hoc, pairwise comparisons. All statistical analyses were performed using SYSTAT® software.

3. Results

3.1. Protein levels of activin A, myostatin, and follistatin in skeletal muscle

ANOVAs performed on activin A and follistatin normalized for total protein, and the ratio of normalized activin A: follistatin, revealed no significant changes with age in either the soleus or extensor digitorum longus muscles. Levels of normalized myostatin showed a slight but non-significant decrease in the EDL with age (Fig. 1A), whereas SOL showed a significant increase in normalized myostatin with age (Fig. 1A). Likewise, the ratio of normalized myostatin: follistatin showed a significant (~40%) decrease with age in EDL (Fig. 1B), but a significant (~four-fold) increase with age in SOL (Fig. 1B). Two-factor ANOVA with age (12 or 24 mo) and muscle (EDL or SOL) as the two factors showed significant age∗muscle interaction effects for both normalized myostatin ($p<.05$) and the myostatin:follistatin ratio ($p<.01$), with myostatin levels decreasing with age in the EDL but increasing with age in SOL.

3.2. Effects of activin A, myostatin, and follistatin on the proliferation and differentiation of primary myoblasts

Overall there was a significant ($P<.01$) age effect for both myostatin and follistatin treatment, in which young cells were more proliferative in response to treatment than older cells. ANOVAs showed significant treatment effects for activin A, myostatin, and follistatin (Fig. 2A). Activin A produced a significant dose–response increase in proliferation in myoblasts from younger mice but not older mice; however, the treatment∗age interaction was not significant. Follistatin also increased proliferation in dose–response manner in young myoblasts, but the effect was attenuated in myoblasts from older mice (Fig. 2B). There was a significant ($p=.001$) treatment∗age interaction effect for follistatin treatment where the treatment effect, approximately two-fold at the highest dose, was much greater in younger myoblasts compared to older cells. Myostatin treatment also increased proliferation in young myoblasts compared to untreated cells, whereas in older myoblasts myostatin decreased proliferation at the low dose (Fig. 2C). There was also a significant ($p<.001$) treatment∗age interaction effect for myostatin, where myostatin treatment increased proliferation in young myoblasts but decreased proliferation in the older cells.

RT-PCR data revealed no marked changes in the expression of differentiation markers MyoD, myogenin, or myosin heavy chain (MHC) with Activin A treatment. MyoD and MHC expression were also unaffected by follistatin treatment of young and aged myoblasts; however, follistatin stimulated a significant increase in myogenin expression in aged but not young myoblasts (Fig. 3A). ANOVAs demonstrated a significant age effect for myogenin expression ($p<.05$) with older cells generally showing higher levels of myogenin expression, and there was also a significant treatment∗age interaction for myogenin expression. Myostatin treatment had no effect on MyoD or myogenin expression in either young or aged myoblasts, but myostatin did produce a significant increase in MHC expression in aged but not young myoblasts (Fig. 3B).

3.3. Protein levels of activin A, myostatin, and follistatin in bone marrow supernatants

Comparisons of normalized protein levels obtained from bone marrow supernatant revealed no significant differences between older and young mice for activin A, follistatin, or the activin A: follistatin ratio. Normalized myostatin is significantly (~80%) increased in mouse bone marrow with increasing age (Fig. 4A), as is the ratio of normalized myostatin: follistatin (+60%; Fig. 4B).

3.4. Effects of activin A, myostatin, and follistatin on the proliferation and differentiation of primary bone marrow stromal cells (BMSCs)

ANOVAs showed a significant ($P<.001$) age effect in each treatment group, with younger BMSCs overall having higher values for proliferation than older BMSCs, irrespective of the treatment (Fig. 5). Proliferation assays showed no effects of activin treatment on either young or old BMSCs (Fig. 5A). Two-factor ANOVA revealed a significant treatment∗age interaction for follistatin, with follistatin having no impact on proliferation in older BMSCs but reducing proliferation in young BMSCs (Fig. 5B). Myostatin significantly decreased BMSC proliferation in both young cells (−15%) and aged cells (−40%); however, this effect was greater in the aged cells, and this treatment∗age interaction was also significant ($P<.05$) for myostatin (Fig. 5C).

Differentiation assays using alizarin red staining to detect mineralization revealed that activin treatment significantly increased mineralization of young and older BMSCs (Fig. 6). The effect of age on mineralization was significant ($P<.001$) for activin, with the

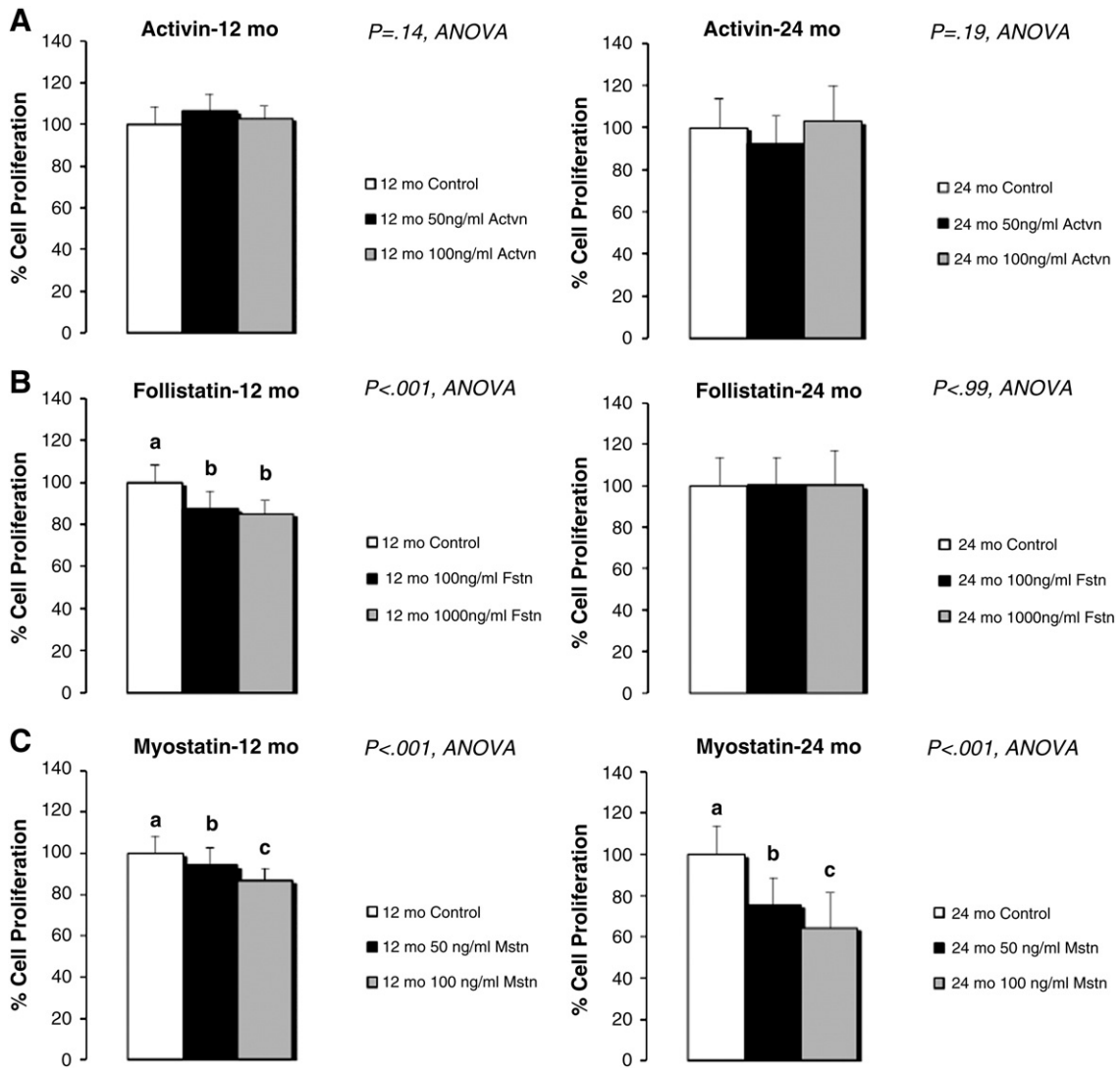


Fig. 5. Results of proliferation assays following treatment of primary bone marrow stromal cells (BMSCs) with activin A (A; activin), follistatin (B; Fstn), and myostatin (C; Mstn). Means with different superscripts differ significantly from one another ($P<.05$). Error bars represent one standard deviation and sample size includes eight replicates per group.

younger cells consistently showing greater mineralization in response to treatment. Treatment effects were less pronounced in aged BMSCs, with the low dose of activin increasing alizarin red staining but the other doses showing no significant effect (Fig. 6C,D). The treatment∗age interaction was significant ($P<.01$) for activin using two-factor ANOVA. In the myostatin experiments, the higher dose of myostatin significantly decreased mineralization in younger cells but not in older cells, and the treatment∗age interaction was also significant for myostatin ($P<.01$). Finally, follistatin treatment did not significantly affect mineralization either young or older BMSCS (Fig. 6).

4. Discussion

The ELISA assays using muscle and bone samples from aged mice provided results that were unexpected for two reasons. First, it is well-known that fast-twitch (type II) muscle fibers atrophy with age (Holloszy et al., 1991) and a number of studies have implicated myostatin in muscle atrophy (McKay et al., 2012). In addition, Hennebry et al. (2009) found that mice lacking myostatin showed an increase in type I fibers and decrease in type II fibers, and blocking myostatin function in vivo using a myostatin antibody has been shown to increases type II fiber size in aging mice (Murphy et al., 2010). The fact that we observed a decline in myostatin levels with age in the predominantly fast-twitch extensor digitorum longus muscle of mice, but a relative increase in myostatin levels in the slow-twitch (type I) soleus, was therefore not anticipated. Nevertheless, it has been shown that mice lacking myostatin lose the same percentage of muscle mass with age as normal mice (Morissette et al., 2009), and muscles composed of different fiber types are all larger in aged myostatin-deficient mice compared to those of same-aged wild-type mice (Jackson et al., 2012). Given that both myostatin levels and the myostatin:follistatin ratio increased with age in soleus muscles of mice compared to extensor digitorum longus, myostatin inhibitors may have a greater impact on preserving muscle mass with age in those muscles composed predominantly of slow- rather than fast-twitch fibers.

The second in vivo finding that was also unexpected was the significant rise in myostatin protein with age in bone marrow supernatants. This was not expected because myostatin is primarily secreted by muscle and not by osteoblasts or marrow stromal cells. Yarasheski et al. (2002) did, however, find that serum myostatin increased with age in older women, and Szulc et al. (2012) found that serum myostatin increased in men until age 57 and then declined. The rise in bone marrow myostatin that we observed with age in mice likely reflects an increase

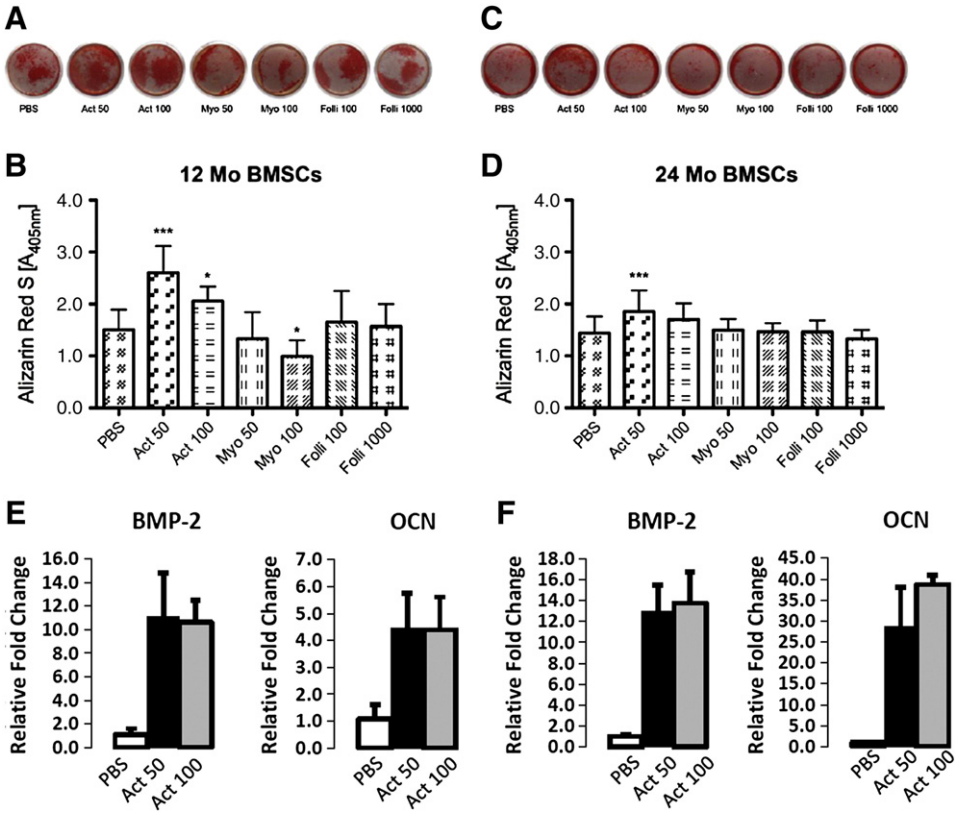


Fig. 6. Alizarin red staining of bone marrow stromal cells cultured in osteogenic conditions (A–D) and PCR data for osteogenic genes (E–F) from BMSCs treated with activin A. Images of wells (A, C) and quantification of staining (B, D) in BMSCs from young mice (A, B; 12 mo BMSCs) and older mice (C, D; 24 mo BMSCs) treated with Activin A (Act), Myostatin (Myo) or Follistatin (Folli) at 50 ng/ml (50), 100 ng/ml (100) or 1000 ng/ml (1000). ***P<.001 and *P<.05 relative to same-aged PBS controls. Error bars represent one standard deviation and sample size includes sixteen replicates per group for panels A–D. PCR data show elevated expression of osteogenic genes BMP-2 and osteocalcin (OCN) in activin-treated cells from mice 12 months of age (E) and mice 24 months of age (F). Error bars represent one standard deviation and four replicates are included per treatment group.

in circulating myostatin levels with age. The elevated concentrations of myostatin in the bone marrow microenvironment of aged mice is significant given the findings discussed below regarding the effects of myostatin on proliferation of bone marrow stromal cells. Moreover, while myostatin was found to be most highly expressed in skeletal muscle progenitors early in development (McPherron et al., 1997), data presented here and elsewhere suggest that myostatin levels may rise again later in life. Thus, the interplay among activin A, myostatin, and follistatin is dynamic and changes across the aging spectrum.

The cell proliferation experiments yielded findings that were consistent with those of previous studies with regard to the effects of age and treatment. First, in both primary myoblasts and BMSCs, age effects were significant regardless of treatment. That is, aged myoblasts and BMSCs were less proliferative overall in response to treatment whether the cells were exposed to activin A, myostatin, or follistatin. This does not appear to be related to expression of ActRIIB, since we have observed using PCR (data not shown) that ActRIIB expression does not differ significantly either between muscle and bone cells of mice at similar ages, or between musculoskeletal tissues of young versus aged mice. Second, in the case of primary myoblasts, all of the ligands generally increased proliferation of young myoblasts at lower doses. Activin A was previously found to suppress embryonic muscle development in chicks (He et al., 2005), and to inhibit proliferation of stem cells derived from adult muscle (Nomura et al., 2008). Myostatin has also been shown previously to be a potent inhibitor of myoblast proliferation (McFarlane et al., 2011), and while myostatin increased proliferation of young myoblasts it decreased proliferation of aged myoblasts. The effects of these ligands on proliferation were quite different in BMSCs, where follistatin and myostatin both suppressed proliferation of young BMSCs, and myostatin significantly suppressed the proliferation of aged BMSCs. These data are consistent with our previous findings showing that BMSCs of mice lacking myostatin were more proliferative in vitro than those from normal mice (Elkasrawy et al., 2011). Probably the most important observation from these in vitro experiments is that, in the case of both aged myoblasts and aged BMSCs, myostatin consistently suppressed cell proliferation. Aged muscle precursors have an impaired capacity for proliferation (Conboy et al., 2003; Machida and Booth, 2004), as do aged BMSCs (Kretlow et al., 2008). Blocking myostatin function in aged animals may therefore have significant potential for improving the repair and regeneration of both muscle and bone by improving the proliferative capacity of both myo- and osteo-progenitor cells.

Findings from our differentiation assays are generally consistent with the idea that myostatin inhibits proliferation and promotes terminal differentiation of aged myoblasts, as myostatin treatment increased myosin heavy chain expression in aged myoblasts. The osteoblast differentiation and mineralization assays revealed that while activin A increased mineralization, myostatin exposure suppressed mineralization in vitro (Fig. 6). These experiments where BMSCs were treated with myostatin are also consistent with our previous studies using BMSCs from myostatin-deficient mice, which showed that $Mstn^{-/-}$ BMSCs had an increased capacity for mineralization in vitro and impaired potential for adipocyte differentiation (Hamrick et al., 2007; Kellum et al., 2009). The activin A differentiation experiments with BMSCs differ somewhat from those of Eijken et al. (2007) who found that activin A treatment strongly inhibited mineralization in osteoblast cultures, whereas follistatin increased mineralization. Our data, on the other hand, showed that osteogenic culture of BMSCs in the presence

of activin A increased mineralization whereas follistatin had no effect on mineralization in either young or old BMSCs. It is likely that our findings differ from those of Eijken et al. (2007) because our experiments examined the effects of activin A and follistatin on the differentiation of osteoprogenitors, whereas Eijken et al. treated differentiated osteoblasts. It is therefore important to acknowledge here that, while our study examined the proliferation and differentiation of progenitor cell populations, ligands of the activin A–myostatin–follistatin system may have different effects on target cells at different stages of development, differentiation, and maturation.

5. Conclusions

Our experiments using aged mice as a model system for investigating the role of activin A, myostatin, and follistatin in musculoskeletal aging suggest that these ligands are likely to be involved in the altered capacity for tissue formation and repair that is observed in aged rodents and humans. Specifically, primary myoblasts and bone marrow stromal cells of aged animals were found to be less proliferative than those of younger animals in response to most of the treatments utilized. Myostatin levels in bone marrow and in soleus muscles increased with age in mice, and myostatin treatment suppressed proliferation of both aged myoblasts and aged BMSCs. Myostatin also inhibited mineralization of BMSCs under osteogenic culture conditions. Together these data suggest that targeting myostatin in aged animals may improve the proliferative capacity of muscle and bone progenitor cells, perhaps enhancing the potential for muscle and bone repair and regeneration in the therapeutic setting.

Acknowledgments

Funding for this research was provided by the Congressionally Directed Medical Research Programs, Department of the Army (CDMRP093619) and the National Institute on Aging (P01 AG036675).

References

Baccarelli, A., Morpurgo, P., Corsi, A., Vaghi, I., Fanelli, M., Cremonesi, G., Vaninetti, S., Beck-Peccoz, P., Spada, A., 2001. Activin A serum levels and aging of the pituitary–gonadal axis: a cross-sectional study in middle-aged and elderly healthy subjects. Exp. Gerontol. 36, 1403–1412.

Conboy, I., Conboy, M.J., Smythe, G., Rando, T.M., 2003. Notch-mediated restoration of regenerative potential to aged muscle. Science 302, 1575–1577.

Dalbo, V.J., Roberts, M.D., Sunderland, K.L., Poole, C.N., Stout, J.R., Beck, T.W., Bemben, M., Kerksick, C.M., 2011. Acute loading and aging effects on myostatin pathway biomarkers in human skeletal muscle after three sequential bouts of resistance exercise. J. Gerontol. A Biol. Sci. Med. Sci. 66, 855–865.

Eijken, M., Swagemakers, S., Koedam, M., Steenbergen, C., Derkx, P., Uitterlinden, A.G., van der Spek, P.J., Visser, J.A., de Jong, F.H., Pols, H.A., van Leeuwen, J.P., 2007. The activin A–follistatin system: potent regulator of human extracellular matrix mineralization. FASEB J. 21, 2949–2960.

Elkasrawy, M.N., Hamrick, M.W., 2010. Myostatin (GDF-8) as a key factor linking muscle mass and bone structure. J. Musculoskelet. Neuronal Interact. 10, 56–63.

Elkasrawy, M.N., Fulzele, S., Bowser, M., Wenger, K., Hamrick, M.W., 2011. Myostatin (GDF-8) inhibits chondrogenesis and chondrocyte proliferation in vitro by suppressing Sox-9 expression. Growth Factors 29, 253–262.

Gilson, H., Schakman, O., Kalista, S., Lause, P., Tsuchida, K., Thissen, J.P., 2009. Follistatin induces muscle hypertrophy through satellite cell proliferation and inhibition of both myostatin and activin. Am. J. Physiol. Endocrinol. Metab. 297, E157–E164.

Gregory, C.A., Gunn, W.G., Peister, A., Prockop, D.J., 2004. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. Anal. Biochem. 329, 77.

Hamrick, M.W., 2011. A role for myokines in muscle–bone interactions. Exerc. Sport Sci. Rev. 39, 43–47.

Hamrick, M.W., 2012. The skeletal muscle secretome: an emerging player in muscle–bone crosstalk. Nature Bonekey, 60, pp. 1–5.

Hamrick, M.W., Ding, K.H., Pennington, C., Chao, Y.J., Wu, Y.D., Howard, B., Immel, D., Borlongan, C., McNeil, P.L., Bollag, W.B., Curl, W.W., Yu, J., Isales, C.M., 2006. Age-related loss of muscle mass and bone strength in mice is associated with a decline in physical activity and serum leptin. Bone 39, 845–853.

Hamrick, M.W., Shi, X., Zhang, W., Pennington, C., Thakore, H., Haque, M., Kang, B., Isales, C.M., Fulzele, S., Wenger, K.H., 2007. Loss of myostatin (GDF-8) function increases osteogenic differentiation of bone marrow-derived stem cells but the osteogenic effect is ablated with unloading. Bone 40, 1544–1553.

He, L., Vichev, K., Macharia, R., Huang, R., Christ, B., Patel, K., Amthor, H., 2005. Activin A inhibits formation of skeletal muscle during chick development. Anat. Embryol. 209, 401–407.

Hennebry, A., Berry, C., Siriett, V., O'Callaghan, P., Chau, L., Watson, T., Sharma, M., Kambadur, R., 2009. Myostatin regulates fiber-type composition of skeletal muscle by regulation MEF2 and MyoD gene expression. Am. J. Physiol. Cell Physiol. 296, C525–C534.

Holloszy, J.O., Chen, M., Cartee, G., Young, J.C., 1991. Skeletal muscle atrophy in old rats: differential changes in the three fiber types. Mech. Aging Dev. 60, 199–213.

Hurwitz, J.M., Santoro, N., 2004. Inhibins, activins and follistatin in the aging female and male. Semin. Reprod. Med. 22, 209–217.

Jackson, M., Luong, D., Vang, D.D., Garikipati, D., Stanton, J., Nelson, O.L., Rodgers, B., 2012. The aging myostatin null phenotype: reduced adiposity, cardiac hypertrophy, enhanced cardiac stress response, and sexual dimorphism. J. Endocrinol. 213, 263–275.

Kellum, E., Starr, H., Arounleut, P., Immel, D., Fulzele, S., Wenger, K., Hamrick, M.W., 2009. Myostatin (GDF-8) deficiency increases fracture callus size, Sox-5 expression, and callus bone volume. Bone 44, 17–23.

Konopka, A., Douglass, M., Kaminsky, L., Jemiolo, B., Trappe, T., Trappe, S., Harper, M., 2010. Molecular adaptations to aerobic exercise training in skeletal muscle of older women. J. Gerontol. A Biol. Sci. Med. Sci. 65, 1201–1207.

Kretlow, J., Jin, Y., Liu, W., Zhang, W., Hong, T.H., Zhou, G., Baggett, L., Mikos, A., Cao, Y., 2008. Donor age and cell passage affects differentiation potential of murine bone marrow-derived stem cells. BMC Cell Biol. 9, 60.

Lee, S.J., Reed, L.A., Davies, M.V., Girgenrath, S., Goad, M.E., Tomkinson, K.N., Wright, J.F., Barker, C., Ehrmantraut, G., Holmstrom, J., Trowell, B., Gertz, B., Jiang, M.S., Sebald, S.M., Matzuk, M., Li, E., Liang, L.F., Quattlebaum, E., Stotish, R.L., Wolfman, N.L., 2005. Regulation of muscle growth by multiple ligands signaling through activin type II receptors. Proc. Natl. Acad. Sci. U. S. A. 102, 18117–18122.

Lee, S.J., Lee, Y.S., Zimmers, T.A., Soleimani, A., Matzuk, M., Tsuchida, K., Cohn, R.D., Barton, E.R., 2010. Regulation of muscle mass by follistatins and activins. Mol. Endocrinol. 24, 1998–2008.

Leger, B., Derave, W., De Bock, K., Hespel, P., Russell, A.P., 2008. Human sarcopenia reveals an increase in SOCS-3 and myostatin and a reduced efficiency of Akt phosphorylation. Rejuvenation Res. 11, 163–175B.

Machida, S., Booth, F.W., 2004. Increased nuclear proteins in muscle satellite cells in aged animals as compared to young growing animals. Exp. Gerontol. 39, 1521–1525.

Marcell, T.J., Harman, S.M., Urban, R.J., Metz, D.D., Rodgers, B.D., Blackman, M.R., 2001. Comparison of GH, IGF-1, and testosterone with mRNA of receptors and myostatin in older men. Am. J. Physiol. Endocrinol. Metab. 281, E1159–E1164.

McFarlane, C., Hui, G., Amanda, W., Lau, H., Lokireddy, S., Xiaojia, G., Mouly, B., Butler-Browne, G., Gluckman, P., Sharma, M., Kambadur, R., 2011. Human myostatin negatively regulates human myoblast growth and differentiation. Am. J. Physiol. Cell Physiol. 301, C195–C203.

McKay, B., Ogborn, D.I., Bellamy, L.M., Tarnopolsky, M.A., Parise, G., 2012. Myostatin is associated with age-related human muscle stem cell dysfunction. FASEB J. 26, 2509–2521.

McPherron, A.C., Lawler, A., Lee, S.J., 1997. Regulation of skeletal muscle mass in mice by a new TGF-β family member. Nature 387, 83–90.

Morissette, M.R., Stricker, C., Rosenberg, M.A., Buranasombati, C., Levitan, E.B., Mittleman, M.A., Rosenzweig, A., 2009. Effects of myostatin deletion in aging mice. Aging Cell 8, 573–583.

Murphy, K., Koopman, R., Naim, T., Leger, B., Trieu, J., Ibebunjo, C., Lynch, G.S., 2010. Antibody-directed myostatin inhibition in 21-mo-old mice reveals novel roles for myostatin signaling in skeletal muscle structure and function. FASEB J. 24, 4433–4442.

Nomura, T., Ueyama, T., Ashihara, E., Tateishi, K., Asada, S., Nakajima, N., Isodono, K., Takahashi, T., Matsubara, H., Oh, H., 2008. Skeletal muscle-derived progenitors capable of differentiating into cardiomyocytes proliferate through myostatin-independent TGF-beta family signaling. Biochem. Biophys. Res. Commun. 365, 863–869.

Pearsall, R.S., Canalis, E., Cornwall-Brady, M., Underwood, K.W., Haigis, B., Ucran, J., Kumar, R., Pobre, E., Grinberg, A., Werner, E.D., Glatt, V., Stadmeyer, L., Smith, D., Seehra, J., Bouxsein, M.L., 2008. A soluble activin type IIA receptor induces bone formation and improves skeletal integrity. Proc. Natl. Acad. Sci. U. S. A. 105, 7082–7087.

Siriett, V., Salerno, M.S., Berry, C., Nicholas, G., Bower, R., Kambadur, R., Sharma, M., 2007. Antagonism of myostatin enhances muscle regeneration during sarcopenia. Mol Ther. 15 (8), 1463–1470.

Szulc, P., Schoppet, M., Goettsch, C., Rauner, M., Dschietzig, T., Chapurlat, R., Hofbauer, L.C., 2012. Endocrine and clinical correlates of myostatin serum concentration in men—the STRAMBO study. J. Clin. Endocrinol. Metab. (Epub ahead of print).

Williams, N.G., Interlichia, J.P., Jackson, M.F., Hwang, D., Cohen, O., Rodgers, B., 2011. Endocrine actions of myostatin: systemic regulation of the IGF and IGF binding protein axis. Endocrinology 152, 172–180.

Yarasheski, K.E., Ghasin, S., Sinha-Hikim, I., Pak-Loduca, J., Gonzalez-Cadavid, N.F., 2002. Serum myostatin-immunoreactive protein is increased in 60–92 year old women and men with muscle wasting. J. Nutr. Health Aging 6, 343–348.

Zhang, W., Ou, G., Hamrick, M., Hill, W., Borke, J., Wenger, K., Chutkan, N., Yu, J., Mi, Q.S., Isales, C.M., et al., 2008. Age-related changes in the osteogenic differentiation potential of mouse bone marrow stromal cells. J. Bone Miner. Res. 23, 1118–1128.

Zhu, J., Li, Y., Shen, W., Qiao, C., Ambrosio, F., Lavasani, M., Nozaki, M., Branca, M., Huard, J., 2007. Relationships between transforming growth factor-β1, myostatin, and decorin. J. Biol. Chem. 282, 25852–25863.

Myostatin (GDF-8) Signaling in Progenitor Cells and Applications to Bone Repair

Moataz N. Elkasrawy, Mark W. Hamrick [1]

[1] Corresponding author:
Professor Mark W. Hamrick, Ph.D.
Department of Cellular Biology & Anatomy
Georgia Health Sciences University
CB1116 Laney Walker Blvd
Augusta, GA 30912 USA
Telephone: +1 706-721-1958
FAX: +1 706-721-6120
Email: mhamrick@georgiahealth.edu

Moataz N. Elkasrawy:
University of Colorado-Denver
School of Dental Medicine
Mail Stop F838
13065 East 17th Avenue, Room 104
Aurora, CO 80045. USA.
Telephone: +1 706-832-5541
FAX: +1 303-724-7066
Email: moataz.elkasrawy@ucdenver.edu

Departmental-Institutional contact:
Ms Sharon Lever
Department of Cellular Biology & Anatomy
Georgia Health Sciences University
Laney Walker Blvd
Augusta, GA 30912 USA
Telephone: +1 706-721-3731
FAX: +1 706-721-6120
Email: slever@georgiahealth.edu

Word count:
5892 words
1 tables
5 figures
68 references

Abbreviations

ActRIIB	type IIB activin receptor
Acvr2b	type IIB activin receptor
Alk4/5	activin-like kinase receptor 4 and 5
BMP-9, -10, 11	bone morphogenetic protein-9, -10, -11
BMSC	bone marrow stromal cell
Dkk1	gene encoding Dickkopf-related protein 1
ERK	extracellular signal-regulated kinase
FLRG	follistatin-related gene
Fzd3	gene encoding the frizzled-3 protein
GDF-8	growth and differentiation factor-8, or myostatin
GDF-11	growth and differentiation factor-11
Gsk3a	gene encoding glycogen synthase kinase 3 alpha
MAPK	mitogen-activated protein kinase
microCT	micro-computed tomography
TGF-β	transforming growth factor-beta
Wnt4	Wingless-type MMTV integration site family, member 4
Wnt5a	Wingless-type MMTV integration site family, member 5a

Abstract

Myostatin is a member of the TGF-β superfamily of growth and differentiation factors and is widely recognized as a potent regulator of skeletal muscle mass and regeneration. It has, however, recently been shown that myostatin is expressed in tissues aside from muscle including fat and tendon. In addition, studies monitoring gene expression in the fracture callus following injury have shown that myostatin is highly expressed in the early phases of fracture healing. Furthermore, other research has demonstrated that mice lacking myostatin show increased bone density and a marked increase in fracture callus size following fibula osteotomy. Yet, the basic mechanisms by which myostatin may impact bone healing after injury have not been fully elucidated. Our in vitro studies using 3D aggregate culture to induce chondrogenic differentiation of bone marrow derived stromal cells (BMSCs) reveal that myostatin suppresses the proliferation and chondrogenic differentiation of BMSCs by altering the expression of Sox-9 and various Wnt-related factors. These in vitro studies suggest that myostatin may directly impair bone regeneration, a hypothesis that is also supported by our recent finding that exogenous myostatin reduces fracture callus bone volume. Together, the data suggest that myostatin may represent a novel therapeutic target for management of orthopedic trauma where both bone and muscle are damaged and, furthermore, that myostatin inhibitors may enhance fracture healing and improve recovery following musculoskeletal injury.

Introduction

Myostatin (GDF-8) was first identified as a new member of the transforming-beta growth factor (TGF-beta) superfamily of growth and differentiation factors that was highly expressed in skeletal muscle progenitors (McPherron et al. 1997). The functional characterization of myostatin using a myostatin-knockout mouse led to the surprising observation that loss of normal myostatin signaling produced a dramatic increase in muscle mass (McPherron et al. 1997). Since the original discovery of myostatin, its role in muscle development and muscle regeneration has been explored using a variety of in vivo and in vitro approaches (Zimmers et al. 2002, Reis-Porszaz et al. 2003, Li et al., 2008, McFarlane et al., 2011). These studies have generally shown that myostatin plays an important role in the proliferation and differentiation of myoblasts, and that inhibiting myostatin expression improves muscle regeneration and increases muscle fiber size and number (Lee 2004, Burks and Cohn 2011).

It is now clear, however, that myostatin is expressed in many tissues aside from muscle (Allen et al. 2008, Mendias et al. 2008), and that it has important functions in cells ranging from fibroblasts to adipocytes. This raises the possibility that myostatin is involved in the differentiation of mesenchymal stem cells (MSCs), since mice lacking myostatin show significant alterations in tissues of mesenchymal origin. Specifically, myostatin deficient mice show decreased body fat (McPherron and Lee 2002, Hamrick et al. 2006), increased bone mineral density (Elkasrawy and Hamrick 2010), and tendons and ligaments that are relatively weak and exhibit cellular hypoplasia (Mendias et al. 2008, Fulzele et al. 2010). In addition, other studies show that the myostatin receptor (Acvr2b, or ActRIIB) is expressed in bone marrow-derived stromal cells (Hamrick et al. 2007), and myostatin can regulate the differentiation of C3H 10T(1/2) mouse mesenchymal multi-potent cells (Rebbapragada et al. 2003, Artaza et al. 2005). Finally, GDF-11, a member of the TGF-β superfamily that is structurally and functionally very similar to myostatin (McPherron et al. 2009), has been shown to decrease chondrogenic and myogenic differentiation of MSCs in micromass cultures (Gamer et al., 2001).

Previous work therefore suggests that myostatin is an important factor in regulating not only the fate of mesenchymal progenitors but also the proliferation, differentiation, and function of mesenchymal derivatives such as adipocytes, chondrocytes, myoblasts, and fibroblasts. These observations have obvious implications for bone repair, since myostatin is also expressed in bone during the early phases of fracture healing (Cho et al. 2002). This chapter reviews the basic biology of myostatin signaling, its role in mediating the proliferation and differentiation of mesenchymal progenitors, and the effects of myostatin and myostatin inhibition on bone repair.

Myostatin Signaling Through the Type IIB Activin Receptor (Acvr2b, or ActRIIB)

Myostatin circulates in a latent form bound to a propeptide, which is cleaved by a BMP1/Tolloid matrix metalloproteinase, releasing the active form capable of binding the type IIB activin receptor. Follistatin and follistatin–related gene (FLRG) are other proteins that can bind and inhibit the activity of myostatin by maintaining its latency (Lee 2004, Tsuchida 2004). Myostatin has previously been shown to the bind the type IIB activin receptor (Acvr2b, or ActRIIB) with high affinity (Lee 2004), with activin itself having a higher affinity for the type IIa activin receptor (Donaldson et al. 1992). Other ligands are, however, also known to bind Acvr2b along with myostatin. In mice these include BMP-9, -10, and -11 along with Inhibinβ and TGF-β1. In human serum the same ligands were found bound to Acvr2b, with the exception of TGF-β1 (Souza et al. 2008).

Acvr2b is a serine/threonine kinase receptor, which recruits and phosphorylates activin-like kinase receptor 5 (ALK5) or ALK4 (Rebbapragada et al 2003). This consequently activates and phosphorylates Smad2/3, which dissociate from the ligand/receptor complex to bind with the co-Smad, Smad4, allowing translocation of the Smad complex to the nucleus where it targets several DNA binding proteins to regulate transcriptional response (Lee 2004) (Fig. 1). Smad4 also activates Smurf-mediated ubiquitination of the ActRIIB/ALK4-5 receptor complex, and promotes its proteosomal degradation (Bradley et al. 2008). Although myostatin, like other

TGF-β family members, is most well known to activate Smad signaling, it can also activate the p38 MAPK and ERK1/2 pathways (Allendorph et al. 2006, Steelman et al. 200, Ekaza and Cabello 2007). The p53 MAPK and ERK1/2 pathways work on promoting the survival of mature muscle synthetia by the action of p53, which blocks apoptosis. ERK1/2 also activates p21, which arrests myoblast proliferation (Bradley et al., 2008). Autocrine myostatin signaling is regulated in part through a negative feedback loop, where myostatin activation of smad2/3 stimulates expression of the inhibitory smad Smad7, which in turn inhibits myostatin signal transduction (Zhu et al. 2004).

A number of studies now provide evidence that myostatin may also signal through the Wnt pathway. For example, Wnt4 expression is significantly elevated in skeletal muscle from myostatin-deficient mice (Steelman et al. 2006), and myostatin enhances nuclear translocation of beta-catenin and formation of the Smad3-beta-catenin-TCF4 complex in human mesenchymal stem cells (Guo et al., 2008). These findings are significant from the perspective of bone healing and regeneration, since components of the Wnt pathway such as Wnt4, Wnt5a, and Wnt5b, are known to be upregulated during the process of fracture repair (Hadjiagyrou et al. 2002; Zhong et al. 2006). In addition, β-catenin-mediated Wnt pathway signaling is also involved in endochondral bone formation (Zhong et al. 2006; Chen et al. 2007a, 2007b). The Wnt pathway ultimately regulates the levels of β-catenin in the cytoplasm and promotes its nuclear translocation, where it acts in conjunction with other transcription factors to promote different gene transcription (Akiyama 2000).

A Role for Myostatin in Regulating Stem Cell Differentiation

A growing body of evidence reveals that myostatin plays a role in regulating myogenesis, adipogenesis and tissue fibrosis by altering the differentiation of progenitor cells of mesenchymal origin. Given the double-muscled phenotype of mice lacking myostatin, it is not surprising that myostatin suppresses cell proliferation during myogenic differentiation of C2C12 progenitors (Taylor et al. 2001, Rios et al. 2001). Myostatin also induces the differentiation of

myofibroblasts in C3H 10T1/2 mesenchymal stem cells (Artaza et al. 2008). In vivo, inhibition of myostatin using an antibody decreases apoptosis and caspase-3 expression in skeletal muscle (Murphy et al. 2010). Myostatin likewise increases proliferation and extracellular matrix synthesis of primary muscle fibroblasts (Li et al 2008), as well as fibroblasts from human knee ligaments (Fulzele et al 2010). These data indicate that myostatin is a pro-fibrotic factor that contributes to the accumulation of collagen-rich fibrous tissue after muscle injury (Zhu et al. 2007).

While myostatin appears to inhibit myogenesis and stimulate fibrosis, it also inhibits adipogenic differentiation of both human bone marrow-derived stromal cells (Guo et al, 2008) and adipogenic differentiation of mouse pre-adipocytes (Kim et al. 2001) and mesenchymal stem cells (Rebbapragada et al. 2003). In human stromal cells, myostatin induced nuclear translocation of beta-catenin, altered the expression of several Wnt/beta-catenin pathway genes, and suppressed expression of the adipogenic transcription factors PPAR gamma and C/EBP alpha (Guo et al 2008). In mouse MSCs and pre-adipocytes, adipogenesis was induced with BMP-7, and myostatin treatment suppressed this BMP7 induced effect (Rebbapragada et al. 2003). The inhibitory effect of myostatin on adipogenesis in these experiments could be reversed by blocking ALK5. In contrast, other studies have shown that myostatin can directly stimulate adipogenesis in C3H10T1/2 cells (Artaza et al. 2005, Feldman et al 2006). These apparently contradictory findings are difficult to reconcile, particularly when congenital absence of myostatin is associated with a dramatic decrease in body fat (Lin et al. 2002, McPherron and Lee 2002). Further investigation into myostatin's role in adipocyte differentiation is clearly needed.

We have previously shown that absence of myostatin increases the osteogenic differentiation of primary bone marrow stromal cells from mice (Hamrick et al. 2007). This finding is perhaps consistent with some of the in vitro experiments above demonstrating that myostatin may favor the adipogenic differentiation of mesenchymal progenitors; hence, absence

of myostatin or myostatin inhibition would be expected to direct MSCs toward the osteogenic or myogenic lineages. This hypothesis is supported by the finding that myostatin inhibitors increase bone formation and bone mass in mice (Bialek et al. 2008, Ferguson et al. 2009), and also improve bone repair following osteotomy (Hamrick et al. 2010). We have also found that myostatin treatment inhibits the chondrogenic differentiation of bone marrow-derived stromal cells by suppressing the expression of Sox-9, and by altering the expression of a number of Wnt-related genes. Specifically, exogenous myostatin reduced the expression of type II collagen and the proliferation of growth plate chondrocytes as well as bone marrow stromal cells in vitro (Elkasrawy et al. 2011a). The Wnt signaling factor Dkk1 is significantly upregulated with myostatin treatment during TGF-β1-induced chondrogenesis, and GSK3A (responsible for beta-catenin instability) is also upregulated, whereas the Wnt ligand binding protein Fzd3 is significantly downregulated with myostatin treatment (Fig. 2). Dkk1 is a molecule that inhibits the Wnt signaling pathway by binding to and antagonizing LRP5/6, forming a ternary complex with LRP6 that induces its rapid endocytosis and removal from the plasma membrane (Mao et al. 2002). GSK3A is another factor that is involved in Wnt signaling, which is a component of the GSK3/Axin/APC proteosomal complex responsible for ubiquitination of β-catenin to prevent its accumulation in the cytoplasm and translocation into the nucleus. Fzd3, a transmembrane protein that can bind Wnt ligands including Wnt5a, and Wnt5a is known to be a molecule that plays a key role in chondrogenesis (Church et al. 2002, Yang et al. 2003), is downregulated in the presence of myostatin during TGF-β1-induced chondrogenesis. In addition, as noted above, GDF-11, a member of the TGF-β superfamily that is structurally very similar to myostatin (McPherron et al. 2009), has been shown to decrease chondrogenic and myogenic differentiation of MSCs in micromass cultures (Gamer et al. 2001).

TGF-β1 is a growth factor that is well known to enhance several key processes in the chondrogenic pathway, including cellular condensation, adhesion, and extracellular matrix production during chondrogenesis (James et al. 2009). TGF-β1 and myostatin can both bind the

same co-receptor, ALK5 (Rebbapragada et al. 2003, Derynck and Feng 1997). We have shown that Sox-9 was down regulated with myostatin treatment, a treatment effect that was also seen with ALK5 inhibition during TGF-β1-induced chondrogenesis (Elkasrawy et al. 2011a). We also showed that GSK3a, responsible for beta-catenin instability, is upregulated with myostatin treatment during TGF-β1-induced chondrogenesis (Fig. 2). These data suggest that myostatin could be competitively inhibiting ALK5-mediated TGF-β1 signaling (Figure 3). TGF-β signaling up-regulates Sox-9 and Wnt5a, and increases nuclear accumulation and stability of beta-catenin during chondrogenesis (Zhou et al., 2004; Kawakami et al., 2006; Lorda-Diez, 2009). Fzd3 is a transmembrane proteins that bind Wnt ligands (Chen and Struhl, 1999). These experimental findings indicate that myostatin might be capable of competitively inhibiting ALK5-mediated TGF-β1 signaling, or alternatively that myostatin may stimulate the expression of certain factors that could actively inhibit TGF-β1, such as Smad7 (e.g. Zhu et al. 2004; Iwai et al. 2008).

Implications for Bone Repair

The first indication that myostatin might play a key role in bone repair and regeneration came from our work in myostatin-knockout mice, which revealed that fracture callus bone volume was significantly increased in mice lacking myostatin following fibular osteotomy (Fig. 4, Kellum et al. 2009). The idea that myostatin might in some way suppress or inhibit bone healing was further supported by another study in which we showed that a myostatin inhibitor (propeptide) increased fracture callus bone volume in mice two weeks following osteotomy (Hamrick et al. 2010a). Fracture healing in rodents involves three key phases that have been well-described by numerous authors: an initial inflammatory phase, a chondrogenic phase, and an osteogenic phase. The in vivo findings in mice lacking myostatin and in mice treated with a myostatin inhibitor are generally supportive of the in vitro data referenced above, suggesting that myostatin acts to suppress chondrogenesis directly (Table 1). Our immunolocalization studies showed that myostatin is highly expressed in the region of the wound blastema 12 and 24 hours following osteotomy, and that exogenous myostatin reduces fracture callus bone volume

(Elkasrawy et al. 2011b). This is one mechanism by which myostatin may influence bone repair (Fig. 5, Table 1), but we believe there are also several others which we describe below.

In addition to suppressing chondrogenesis during fracture healing, myostatin may also mediate the differentiation of muscle-derived progenitors that contribute to callus formation. It has been recognized for decades that the muscle bed itself plays a crucial role in fracture healing (Pritchard and Ruzicka 1950), and that cells derived from muscle may in fact migrate into the site of injury and promote bone healing (Schindeler et al. 2009, Liu et al., 2010). Cells harvested from muscle after exposure to an adjacent fracture are highly osteogenic and form bone nodules in vitro (Glass et al. 2011). Thus, muscle not only facilitates bone repair by providing a vascular bed and perhaps trophic factors (see below), but it is a possible source of progenitor cells that can differentiate into osteoblasts, further improving bone healing (Glass et al. 2011). Given that myostatin is a potent factor that can mediate the differentiation of muscle-derived cells, it is likely that elevated levels of myostatin following injury may drive muscle-derived stromal cells toward a myofibroblast lineage rather than toward an osteogenic fate. In this way local or systemic myostatin could inhibit bone healing by limiting the supply of osteoprogenitors available from the pool of muscle-derived stromal cells (Fig. 5).

A third manner in which myostatin may alter bone healing is by regulating muscle regeneration and hence the secretion of muscle-derived paracrine factors (“myokines”; Hamrick 2011). Studies in which skeletal muscle is transplanted into cardiac muscle support the notion that myofibers secrete a number of paracrine factors that are not only osteogenic (Hamrick 2011) but also promote the survival of neighboring muscle fibers (“paracrine theory of transplantation”; Perez-Ilzarbe et al. 2008). Factors that are actively secreted by myotubes in vitro include insulin-like growth factor 1 (IGF-1), osteonectin (SPARC), and basic fibroblast growth factor (FGF-2) (Hamrick, 2011). All of these factors have been observed to increase bone formation when injected peripherally, and deficiency of these factors is associated with low bone mass (Hamrick, 2011). Elevated levels of myostatin impair muscle regeneration and

induce muscle atrophy, which would in turn be expected to reduce the secretion of factors normally produced by healthy myofibers such as IGF-1 and FGF-2 (Hamrick et al, 2010b). Recently it was also shown that mice lacking myostatin have elevated serum levels of IGF-1, and that this was associated with elevated expression of IGF-1 in the liver (Williams et al. 2011). Thus, elevated secretion of myostatin, which is known to occur with disuse, infection, burns, or AIDS- and cancer-related cachexia, may indirectly impair bone healing and bone formation by decreasing circulating levels of liver-derived IGF-1 (Williams et al. 2010).

Summary

Since the discovery of myostatin in 1997 our understanding of its biology and function has grown considerably so that we now recognize a broad range of potential roles for myostatin in musculoskeletal growth, development, and regeneration. These include effects of myostatin on tendon and ligament development and healing (Mendias et al. 2008, Eliasson et al. 2009, Fulzele et al. 2010), adipogenesis and fat deposition (McPherron and Lee 2002, Hamrick et al. 2006), and bone density and bone healing (Morissette et al. 2009; Kellum et al. 2009), in addition to the well-documented effects of myostatin on muscle mass and regeneration (Lee 2004). These and other in vivo and in vitro studies now point to a role for myostatin in the proliferation and differentiation of mesenchymal progenitors. These effects are mediated by TGF-beta signaling through the type IIB activin receptor, involving phosphorylation of Smad2/3, but our own work in chondrocytes and the work of others in MSCs and myoblasts suggest that myostatin also signals through the Wnt pathway. Various myostatin inhibitors have, not surprisingly, been observed to increase muscle mass, decrease fat mass, and increase bone formation. These findings suggest that myostatin represents a potential therapeutic target for improving tissue repair and regeneration in musculoskeletal diseases and following musculoskeletal injury.

Acknowledgments

Many of the findings discussed in this chapter are derived from our research on myostatin which has been supported by the National Institutes of Health (AR049717), the Office of Naval Research (N000140810197), and the Department of the Army (USAMRMC PR093619). We are grateful to Drs. Paul Yaworsky, Alexandra McPherron, and Li-Fang Liang for many helpful discussions regarding the biology of myostatin. Ethan Kellum, Phonepasong Arounleut, Matthew Bowser, David Immel, Craig Byron, Penny Roon, Donna Kumiski, and Cathy Pennington provided valuable technical assistance and support for many of the studies reviewed in this paper.

Summary Points

- Myostatin, also known as growth and differentiation factor-8 (GDF-8), was discovered in 1997.
- Myostatin plays a number of roles in musculoskeletal growth, development, and regeneration.
- These include effects of myostatin on tendon and ligament development and healing, adipogenesis and fat deposition, and bone density and bone repair.
- Blocking myostatin signaling is well-known to increase muscle mass and improve muscle regeneration.

Dictionary

Wnt pathway: The wnt signaling pathway is most well known for its role in regulating cell fate and cell-cell interactions during embryonic development in both vertebrates and invertebrates. The term “wnt” refers to the name of the gene implicated in the wingless mutation in fruit flies (Drosophila).

Endochondral ossification: Long bones such as the humerus and femur of the mammalian skeleton first form as condensations of cartilage, which then undergo a process of ossification to form a bony skeleton. This process is referred to as endochondral ossification, and a similar process occurs during bone healing, where a “soft” callus of cartilage is replaced by a “hard” bony callus.

Bone marrow stromal cells: Bone marrow includes a variety of cell types, including cells that can give rise to tissue types of mesodermal origin (e.g., muscle, fat, bone). These cells in bone marrow are referred to as bone marrow stromal cells.

Myokines: It is now known that muscle can secrete a number of different growth factors and cytokines that can affect other organs, and these muscle-derived factors are referred to as myokines.

References

Akiyama, T. 2000. Wnt/beta-catenin signaling. Cytokine Growth Factor Rev. 11:273-82.

Allen, D.L., and A. Cleary, K. Speaker, S.F. Lindsay, J. Uyenishi, J.M. Reed, M.C. Madden and R.S. Mehan. 2008. Myostatin, activin receptor IIB, and follistatin-like-3 gene expression are altered in adipose tissue and skeletal muscle of obese mice. Am. J. Physiol. Endocrinol. Metab. 294: E918-27.

Allendorph, G. and W. Vale and S. Choe. 2006. Structure of the ternary signaling complex of a TGF-beta superfamily member. Proc. Natl. Acad. Sci. USA 103: 7643-7648.

Artaza, J. and S. Bhasin, T. Magee, S. Reisz-Porszasz, R. Shen, N. Groome, M. Fareez and N. Gonzalez-Cadavid. 2005. Myostatin inhibits myogenesis and promotes adipogenesis in C3H 10T(1/2) mesenchymal multi-potent cells. Endocrinology 146:3547-57.

Bialek, P. and J. Parkington, L. Warner, M. St. Andre, L. Jian, D. Gavin, C. Wallace, J. Zhang, G. Yan, A. Root, H. Seeherman and P. Yaworsky. 2008. Mice treated with a myostatin/GDF-8 decoy receptor, ActRIIB-Fc, exhibit a tremendous increase in bone mass. Bone 42: S46.

Bradley, L. and P. Yaworsky and F. Walsh. 2008. Myostatin as a therapeutic target for musculoskeletal disease. Cell. Mol. Life. Sci. 65:2119-24.

Burks, T. and R. Cohn. 2011. Role of TGF-beta signaling in inherited and acquired myopathies. Skelet. Muscle 1:19.

Chen, Y. and H. C. Whetstone, A. Youn, P. Nadesan, E. C. Chow, A. C. Lin and B. A. Alman. 2007a. Beta-catenin signaling pathway is crucial for bone morphogenetic protein 2 to induce new bone formation. J. Biol.Chem. 282:526-33.

Chen, Y. and H. C. Whetstone, A. C. Lin, P. Nadesan, Q. Wei, R. Poon, and B. A. Alman. 2007b. Beta-catenin signaling plays a disparate role in different phases of fracture repair: implications for therapy to improve bone healing. PLoS Med 4(7):e249.

Chen, Y.and B. A. Alman. 2009. Wnt pathway, an essential role in bone regeneration. J. Cell Biochem. 106:353-62.

Chen, C. and G. Struhl. 1999. Wingless transduction by the Frizzled and Frizzled2 proteins of Drosophila. Development 126: 5441-42.

Cho, T. and L. Gerstenfeld and T. Einhorn. 2002. Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. J. Bone Miner Res. 17: 513-520.

Church, V. and T. Nohno, C. Linker, C. Marcelle and P. Francis-West. 2002. Wnt regulation of chondrocyte differentiation. J. Cell Sci. 115: 4809.

Derynck, R. and X. Feng. 1997. TGF-beta receptor signaling. Biochim. Biophys. Acta. 1333: F105-50.

Donaldson, C. and L. Mathews and W. W. Vale. 1992. Molecular cloning and binding properties of the human type II activin receptor. Biochem. Biophys. Res. Commun. 194: 310-6.

Ekaza, D. and G. Cabello. 2007. The myostatin gene: physiology and pharmacological relevance. Curr. Opin. Pharmacol 7:310-5.

Eliasson, P. and T. Andersson, J. Kulas, P. Seemann, P. Aspenberg. 2009. Myostatin in tendon maintenance and repair. Growth Factors 27: 247-54.

Elkasrawy, M.N. and M. W. Hamrick. 2010. Myostatin (GDF-8) as a key factor linking muscle mass and bone structure. J. Musculoskelet Neuronal Interact. 10: 56-63.

Elkasrawy, M.N. and S. Fulzele, M. Bowser, K. Wenger and M. W. Hamrick. 2011a. Myostatin (GDF-8) inhibits chondrogenesis and chondrocyte proliferation in vitro by suppressing Sox-9 expression. Growth Factors. July 15 [ePub ahead of print].

Elkasrawy M., and D. Immel, L-F. Liang, X. Wen and M.W. Hamrick. 2011b. Immunolocalization of myostatin (GDF-8) following musculoskeletal injury and the effects of exogenous myostatin on muscle and bone healing. J Histochem. Cytochem. [in press].

Feldman, B.J. and R. Streeper, R. Farese, and K. Yamamoto. 2006. Myostatin modulates adipogenesis to generate adipocytes with favorable metabolic effects. Proc. Natl. Acad. Sci. USA 103: 15675-80.

Ferguson, V. and R. Paietta, L. Stodieck, A. Hanson, M. Young and T. Bateman. et al. 2009. Inhibiting myostatin prevents microgravity-associated bone loss in mice. J. Bone Miner. Res. 24 (supp 1): 1288.

Fulzele, S. and P. Arounleut, M. Cain, S. Herberg, M. Hunter, K. Wenger and M. W. Hamrick. 2010. Role of myostatin (GDF-8) signaling in the human anterior cruciate ligament. J. Orthop Res. 28: 1113-1118.

Gamer, L.W. and K. A. Cox, C. Small and V. Rosen. 2001. Gdf11 is a negative regulator of chondrogenesis and myogenesis in the developing chick limb. Dev. Biol. 229:407-20.

Glass, G. and J. Chan, A. Freidin, M. Feldmann, N. Horwood and J. Nanchacal. 2011. TNF-alpha promotes fracture repair by augmenting the recruitment and differentiation of muscle-derived stromal cells. Proc. Natl. Acad. Sci .USA 108: 1585-90.

Guo, W. et al. 2008. The effects of myostatin on adipogenic differentiation of human bone marrow-derived mesenchymal stem cells are mediated through cross-communication between Smad3 and Wnt/beta-catenin signaling pathways. J Biol. Chem. 283: 9136-9145.

Hadjiargyrou, M. and F. Lombardo, S. Zhao, W. Ahrens, J. Joo, H. Ahn, M. Jurman and D. W. White. Rubin CT 2002 Transcriptional profiling of bone regeneration. Insight into the molecular complexity of wound repair. J. Biol. Chem. 277:30177-82.

Hamrick, M. W. and C. Pennington, C. Webb, and C. M. Isales. 2006. Resistance to body fat gain in ‘double-muscled’ mice fed a high-fat diet. Int. J. Obes. 30: 868-870.

Hamrick, M.W. and X. Shi, W. Zhang, C. Pennington, B. Kang, H. Thakore, M. Haque, C. M. Isales, S. Fulzele and K. Wenger. 2007. Loss of myostatin function increases osteogenic differentiation of bone marrow-derived mesenchymal stem cells but the osteogenic effect is ablated with unloading. Bone 40: 1544-1553.

Hamrick, M.W., P. Arounleut, E. Kellum, M. Cain, D. Immel and L. Liang. 2010a. Recombinant myostatin (GDF-8) propeptide enhances the repair and regeneration of both muscle and bone in a model of deep penetrant musculoskeletal injury. Journal of Trauma 69: 579-83.

Hamrick, M.W. and P. L. McNeil and S. L. Patterson. 2010b. Role of muscle-derived growth factors in bone formation. J Musculoskel. Neuronal Interact. 10: 64-70.

Hamrick, M.W. 2011. A role for myokines in muscle-bone interactions. Exercise & Sports Science Reviews 39: 43-47.

Iwai, T. and J. Murai, H. Yoshikawa, and N. Tsumaki. 2008. Smad7 inhibits chondrocyte differentiation at multiple steps during endochondral bone formation and down-regulates p38 MAPK pathways. J. Biol. Chem. 283: 27154-64.

James, A.W. and Y. Xu, J. K. Lee, R. Wang and M. T. Longaker. 2009. Differential effects of TGF-beta1 and TGF-beta3 on chondrogenesis in posterofrontal cranial suture-derived mesenchymal cells in vitro. Plast. Reconstr. Surg. 123:31-43.

Kawakami, Y. and J. Rodriguez-Leon and J. C. Izpisua Belmonte. 2006. The role of TGFbetas and Sox9 during limb chondrogenesis. Curr. Opin. Cell Biol. 18:723-9.

Kellum, E. and H. Starr, D. Immel, P. Arounleut, S. Fulzele, K. Wenger and M. W. Hamrick. 2009. Myostatin (GDF-8) deficiency increases fracture callus size, Sox-5 expression, and callus bone volume. Bone 44: 17-23.

Kim, H. and L. Liang, R. Dean, D. Hausman, D. Hartzell and C. Baile. 2001. Inhibition of preadipocyte differentiation by myostatin treatment in 3T3-L1 cultures. Biochem. Biophys. Res. Commun. 281: 902-906.

Li Z. Kollias H. and K. R. Wagner. 2008. Myostatin directly regulates skeletal muscle fibrosis. J. Biol. Chem. 283:19371-1938.

Lin, J., Arnold, H. and M. Della-Fera, M. Azain, D. Hartzell, C. A. Baile. 2002. Myostatin knockout in mice increases myogenesis and decreases adipogenesis. Biochem Biophys Res Commun 291: 701-6.

Liu, R., and A. Schindeler, and D. Little. 2010. The potential role of muscle in bone repair. J. Musculoskel. Neuronal Interact. 10: 71-6.

Lorda-Diez, C.I. and J. A. Montero, C. Martinez-Cue, J. A. Garcia-Porrero, J. M. Hurle. 2009. Transforming growth factors beta coordinate cartilage and tendon differentiation in the developing limb mesenchyme. J. Biol. Chem. 284(43):29988-96.

Mao, B. and W. Wu, G. Davidson, J. Marhold, M. Li, B. M. Mechler, H. Delius, D. Hoppe, P. Stannek, C. Walter, A. Glinka and C. Niehrs. 2002. Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. Nature 417: 664.

McPherron, A.C. and A. M. Lawler and S-J Lee. 1997. Regulation of skeletal muscle mass in mice by a new TGF-ß superfamily member. Nature 387: 83-90.

McPherron, A.C. and S-J Lee. 2002. Suppression of body fat accumulation in myostatin-deficient mice. J. Clin. Invest. 109: 595-601.

McPherron , A.C. and T. V. Huynh and S. J. Lee. 2009. Redundancy of myostatin and growth/differentiation factor 11 function. BMC Dev. Biol. 9:24.

Lee, S.J. 2004. Regulation of muscle mass by myostatin. Annu. Rev. Cell Dev. Biol. 20:61-86.

Mendias, C. and K. Bakhurin and J. Faulkner. 2008. Tendons of myostatin-deficient mice are small, brittle, and hypocellular. Proc. Natl. Acad. Sci. USA. 105: 388-393.

McFarlane. C. and G. Hui, W. Amanda, H. Lau, S. Lokireddy, G. Xiaojia, V. Mouly, G. Butler-Browne, P. Gluckman, M. Sharma and R. Kambadur. 2011. Human myostatin negatively regulates human myoblast growth and differentiation. Am. J. Physiol. Cell. Physiol. 301: C195-203.

Morissette, M., and J. Stricker, M. Rosenberg, C. Buranasombati, E. Levitan, M. Mittleman, and A. Rosenzweig. 2009. Effects of myostatin deletion in aging mice. Aging Cell 8: 573-83.

Perez-Ilzarbe, M., and Agbulut, B. Pelacho, C. Ciorba, E. San Jose-Eneriz, M. Desnos, A. Hagege, P. Aranda, E. Andreu, P. Menasche and F. Prosper. 2008. Characterization of the paracrine effects of human skeletal myoblasts transplanted in infracted myocardium. Eur. J. Heart Fail. 10: 1065-72.

Phiel, C.J. and P. S. Klein. 2001 Molecular targets of lithium action. Annu. Rev. Pharmacol Toxicol 41:789-813.

Pritchard, J. and A. Ruzicka. 1950. Comparison of fracture repair in the frog, lizard, and rat. J Anat. 84: 236-261.

Rebbapragada, A. and H. Benchabane, J. Wrana, A. Celeste and L. Attisano. 2003. Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. Mol. Cell. Biol. 23: 7230-7242.

Reisz-Porszasz, S. and S. Bhasin, J. Artaza and R. Shen et al. 2003. Lower skeletal muscle mass in male transgenic mice with muscle-specific overexpression of myostatin. Am. J. Physiol. Endocrinol Metab. 285: E876-88.

Rios, R., and I. Carniero, V. Arce, and J. Devesa. 2001. Myostatin regulates cell survival during C2C12 myogenesis. Biochem. Biophys. Res. Commun. 280: 561-66.

Schindeler, A., and R. Liu and D. Little. 2009. The contribution of different cell lineages to bone repair: exploring a role for muscle stem cells. Differentiation 77: 12-18.

Souza, T. et al. 2008. Proteomic identification and functional validation of activins and bone morphogenetic protein 11 as candidate novel muscle mass regulators. Mol. Endocrinol 22: 2689-702.

Steelman, C.A. and J. C. Recknor, D. Nettleton and J. M. Reecy. 2006. Transcriptional profiling of myostatin-knockout mice implicates Wnt signaling in postnatal skeletal muscle growth and hypertrophy. Faseb J 20(3):580-2.

Taylor, W.E., and S. Bhasin, J. Artaza, F. Byhower, M. Azam, D. Willard, F. Kull, and N. Gonzalez-Cadavid. 2001. Am. J. Physiol. Endocrinol. Metab. E221-8.

Tsuchida, K. 2004. Activins, myostatin and related TGF-beta family members as novel therapeutic targets for endocrine, metabolic and immune disorders. Curr Drug Targets Immune Endocr. Metabol. Disord. 4:157-66.

Williams, N. and J. Interlichia, M. Jackson, D. Hwang, P. Cohen and B. Rodgers. 2011. Endocrine actions of myostatin: systemic regulation of the IGF and IGF binding protein axis. Endocrinology 152: 172-80.

Yang, Y., Topol, L., Lee, H., Wu, J. 2003. Wnt5a and Wnt5b exhibit distinct activities in coordinating chondrocyte proliferation and differentiation. Development 130: 1003.

Zhang, F. and C. J. Phiel, L. Spece, N. Gurvic and P. S. Klein. 2003. Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium. Evidence for autoregulation of GSK-3. J. Biol. Chem. 278:33067-77.

Zhong,N. and R. P. Gersch and M. Hadjiargyrou. 2006. Wnt signaling activation during bone regeneration and the role of Dishevelled in chondrocyte proliferation and differentiation. Bone 39:5-16.

Zhou, S. and K. Eid and J. Glowacki. 2004. Cooperation between TGF-beta and Wnt pathways during chondrocyte and adipocyte differentiation of human marrow stromal cells. J. Bone Miner Res. 19:463-70.

Zhu, X. and S. Topouzi, L. F. Liang and R. L. Stotish. 2004. Myostatin signaling through Smad2, Smad3 and Smad4 is regulated by the inhibitory Smad7 by a negative feedback mechanism. Cytokine 26: 262-72.

Zhu. J. and Y. Li, W. Shen, C. Qiao, F. Ambrosio, M. Lavasani, M. Nozak, M. Branca, J. Huard. 2007. Relationships between transforming growth factor-beta1, myostatin, and decorin: implications for skeletal muscle fibrosis. J. Biol. Chem. 282: 25852-63.

Zimmers, T.A. and M. V. Davie, L. G. Koniaris, P. Haynes, A. F. Esquela, K. N. Tomkinson, A. C. McPherron, N. M. Wolfman and S. J. Lee. 2002. Induction of cachexia in mice by systemically administered myostatin. Science 296: 1486-1488.

Table 1. Key Facts Related to Myostatin and Bone Healing

1. The receptor for myostatin is expressed in bone marrow stromal cells, and chondrocytes as well as muscle cells express myostatin.
2. Following traumatic musculoskeletal injury, myostatin is most highly expressed by injured muscle cells in the first 24-48 hours following the injury.
3. Congenital absence of myostatin increases the volume of cartilage and bone in the fracture callus, whereas delivery of exogenous myostatin impairs muscle and bone healing.
4. In vitro experiments show that myostatin can inhibit the chondrogenic differentiation of bone marrow stromal cells.
5. Blocking myostatin signaling immediately following musculoskeletal injury is therefore a potential therapeutic strategy for improving the healing of both muscle and bone.

Legend to Table 1. This table lists key facts that are related to the role of myostatin in bone repair, including direct roles for myostatin on osteo- and chondroprogenitors, and indirect roles mediated by muscle-derived factors.

Figure Caption Titles and Legends

Figure 1 Title. The myostatin signaling pathway.

Figure 1 Legend. Myostatin (M) binds the type IIB activin receptor (ActRIIB) which recruits the type I co-receptor ALK5, phosphorylating Smad2/3 which translocate to the nucleus where they regulate target gene expression. A number of factors thought to be involved in the proliferation and differentiation of mesenchymal stem cells are regulated through this TGF-beta signaling pathway.

Figure 2 Title. Effects of myostatin treatment on gene expression.

Figure 2 Legend: Exposure of bone marrow-derived mesenchymal stem cells to exogenous myostatin alters the expression of several factors involved in the Wnt signaling pathway.

Figure 3 Title. Hypothetical model showing mechanisms by which myostatin may mediate TGF-beta induced chondrogenesis.

Figure 3 Legend. TGF-beta 1 and myostatin compete for recruitment of ALK5. TGF-beta stimulates Wnt5a activation of the chondrogenic cascade, whereas myostatin may attenuate this effect.

Figure 4 Title. Effects of myostatin deficiency on fracture healing.

Figure 4 Legend. MicroCT images of the fibula fracture callus in normal mice (left two panels) and mice lacking myostatin (right two panels).

Figure 5 Title. General model summarizing effects of myostatin inhibition (left) and myostatin expression (right) on musculoskeletal repair and regeneration.

Figure 5 Legend. Blocking myostatin signaling following injury of muscle and bone is likely to enhance bone healing in two ways (left side of figure): directly, because suppression of

myostatin activity will enhance chondrogenesis and indirectly, because enhancing muscle regeneration is likely to restore the normal secretion of paracrine, osteogenic trophic factors (e.g., IGF-1) from muscle. Myostatin expression following musculoskeletal injury inhibits bone healing in two ways (right side of figure), first by suppressing chondrogenesis directly and second by increasing fibrosis in injured muscle thereby reducing secretion of osteogenic paracrine factors from muscle tissue.

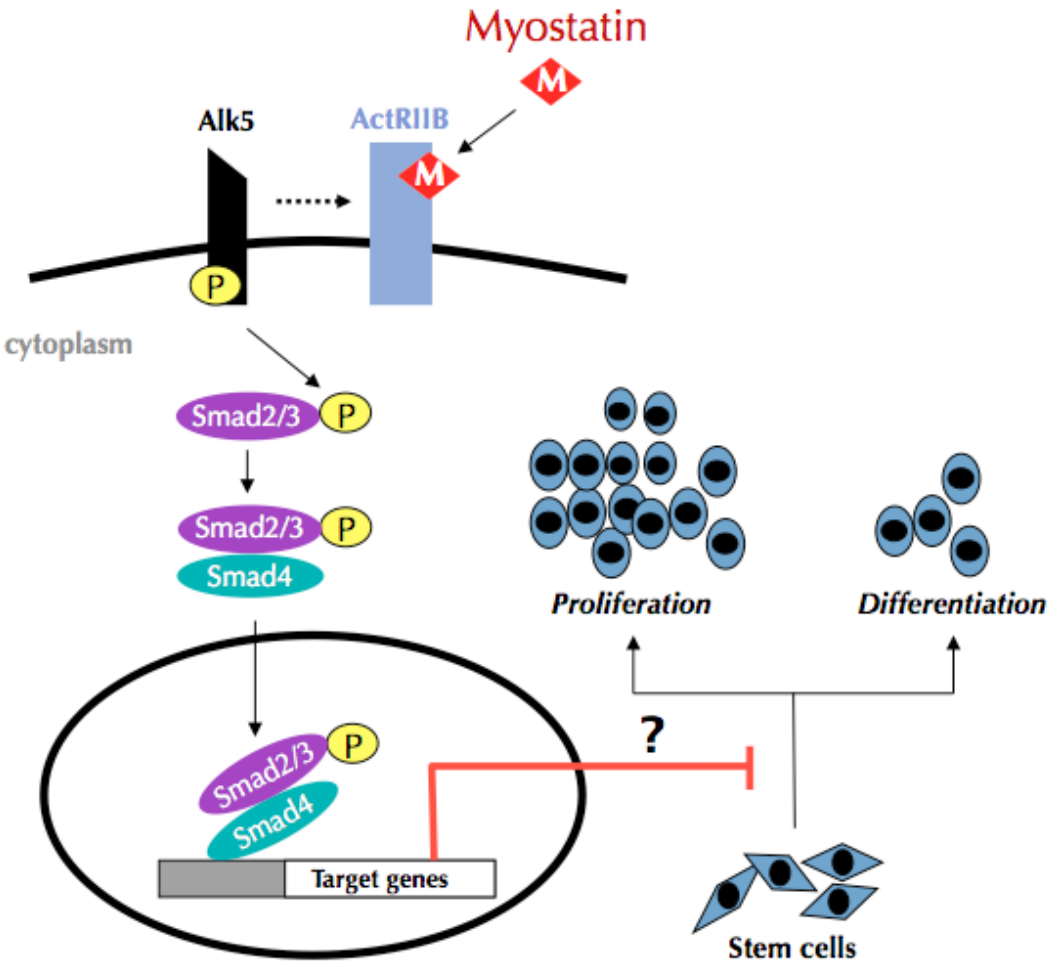

Myostatin
M
Alk5
ActRIIB
M
P
cytoplasm
Smad2/3
P
Smad2/3
P
Smad4
Proliferation
Differentiation
P
Smad2/3
Smad4
?
Target genes
Stem cells

Figure 1

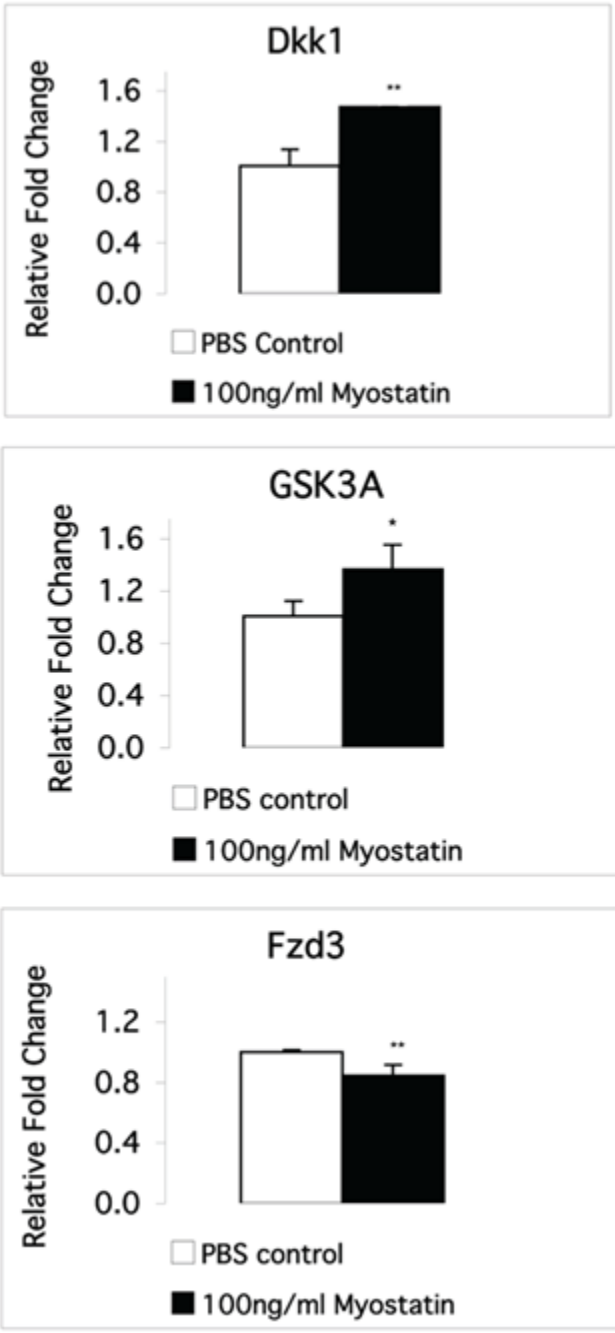

Dkk1
Relative Fold Change
1.6
1.2
0.8
0.4
0.0
**
PBS Control
100ng/ml Myostatin
GSK3A
Relative Fold Change
1.6
1.2
0.8
0.4
0.0
*
PBS control
100ng/ml Myostatin
Fzd3
Relative Fold Change
1.2
0.8
0.4
0.0
**
PBS control
100ng/ml Myostatin

Figure 2

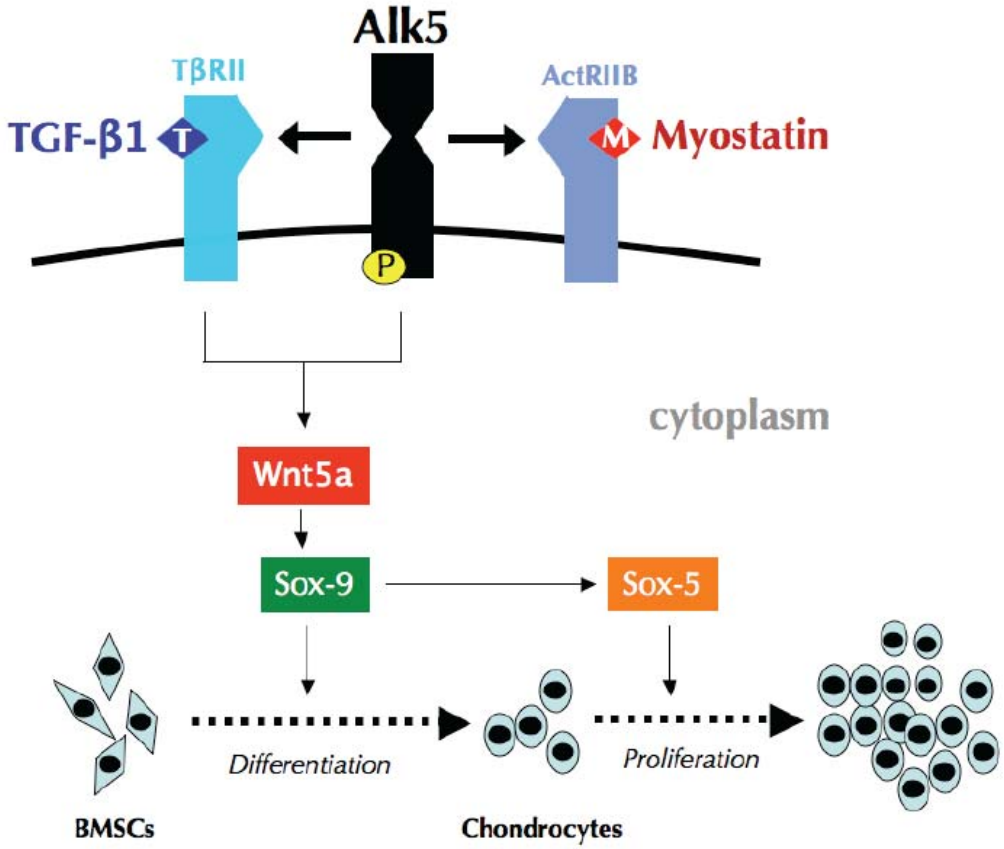

Alk5
TβRII
ActRIIB
TGF-β1
T
M
Myostatin
P
cytoplasm
Wnt5a
Sox-9
Sox-5
Differentiation
Proliferation
BMSCs
Chondrocytes

Figure 3

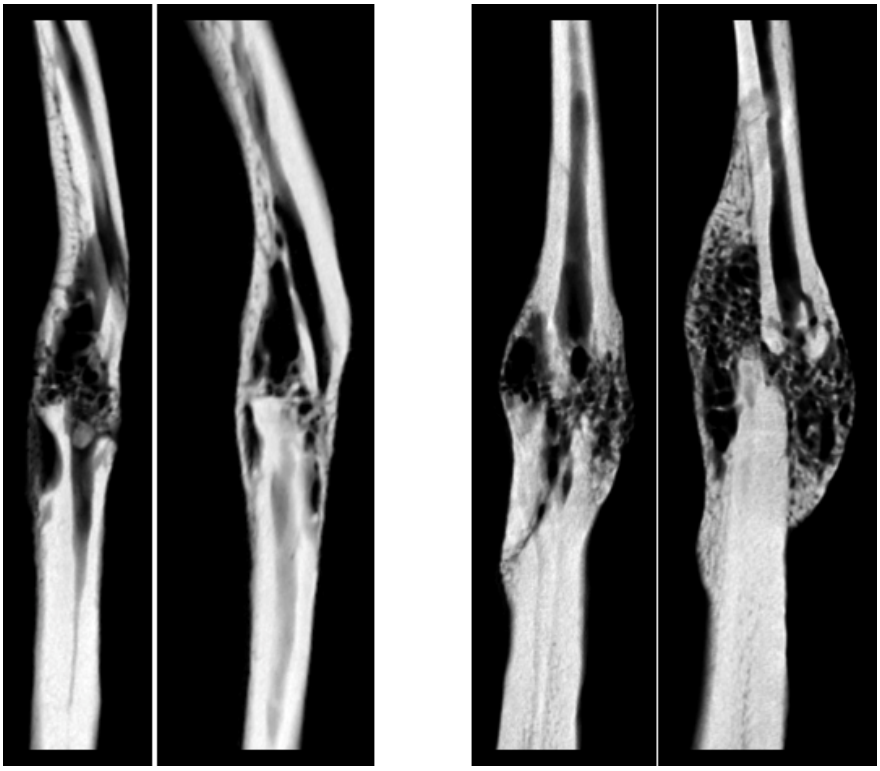


Figure 4

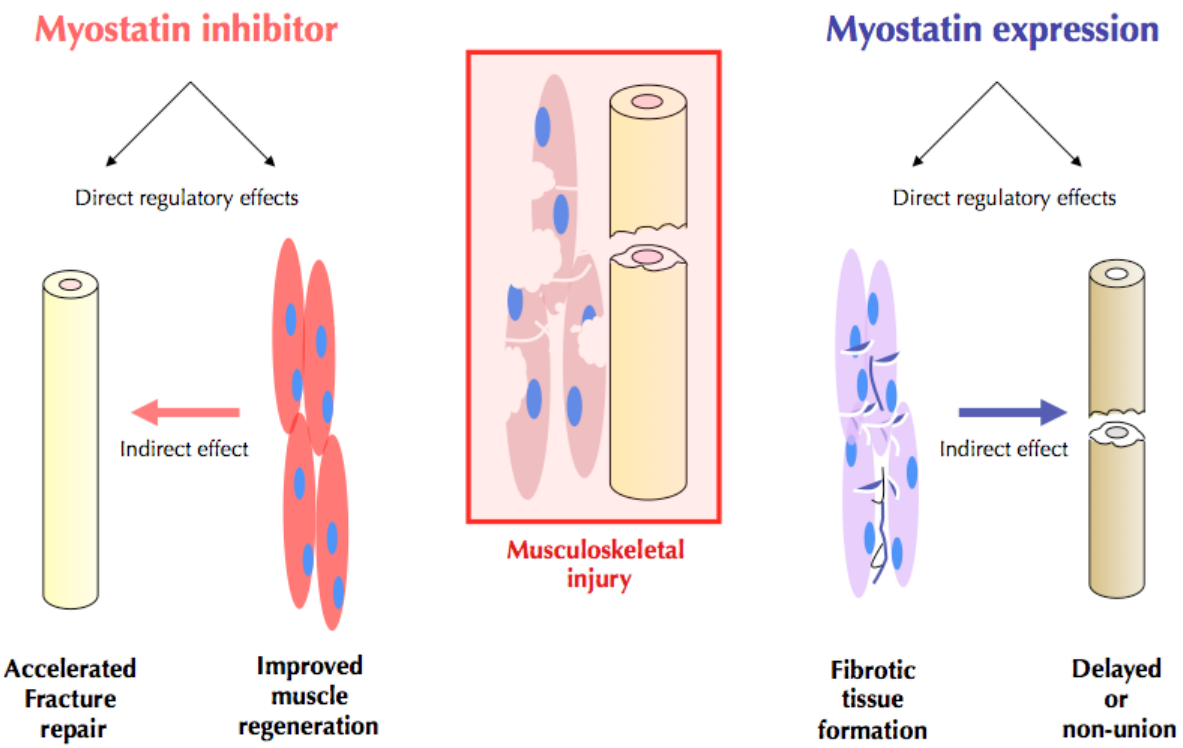

Myostatin inhibitor
Direct regulatory effects
Indirect effect
Accelerated Fracture repair
Improved muscle regeneration
Musculoskeletal injury
Myostatin expression
Direct regulatory effects
Indirect effect
Fibrotic tissue formation
Delayed or non-union

Figure 5

A Myostatin Inhibitor (Propeptide-Fc) Increases Muscle Mass and Muscle Fiber Size in Aged Mice but Does not Increase Bone Density or Bone Strength

Phonepasong Arounleut[1], Peter Bialek[2], Li-Fang Liang[3], Sunil Upadhyay[1], Sadanand Fulzele[1], Maribeth Johnson[1], Mohammed Elsalanty[1], Carlos M. Isales[1], Mark W. Hamrick[1]*

[1]Georgia Regents University, Augusta, GA 30912, USA

[2]Pfizer Inc., Cambridge, MA 02139, USA

[3]The Tauri Group, Alexandria, VA 22310, USA

Pages: 20 (including references and figure captions)

Tables: 3

Figures: 4

Running Title: Effects of Myostatin Inhibitor on Aging Muscle & Bone

Key Words: sarcopenia; osteoporosis; fractures; anabolic therapy

*Address for correspondence and reprints:

Mark W. Hamrick
Department of Cellular Biology & Anatomy
Laney Walker Blvd. CB2915
Georgia Regents University (*formerly Georgia Health Sciences University*)
Augusta, GA 30912 USA
PH: 706-721-1958
FAX: 706-721-6120
EMAIL: mhamrick@gru.edu

Abstract

Loss of muscle and bone mass with age are significant contributors to falls and fractures among the elderly. Myostatin deficiency is associated with increased muscle mass in mice, dogs, cows, sheep and humans, and mice lacking myostatin have been observed to show increased bone density in the limb, spine, and jaw. Transgenic overexpression of myostatin propeptide, which binds to and inhibits the active myostatin ligand, also increases muscle mass and bone density in mice. We therefore sought to test the hypothesis that in vivo inhibition of myostatin using an injectable myostatin propeptide (GDF8 propeptide-Fc) would increase both muscle mass and bone density in aged (24 mo) mice. Mice were injected weekly (20 mg/kg body weight) with recombinant myostatin propeptide-Fc (PRO) or vehicle (VEH; saline) for four weeks. There was no difference in body weight between the two groups at the end of the treatment period, but PRO treatment significantly increased mass of the tibialis anterior muscle (+7%) and increased muscle fiber diameter of the extensor digitorum longus (+16%) and soleus (+6%) muscles compared to VEH treatment. Bone volume relative to total volume (BV/TV) of the femur calculated by microCT did not differ significantly between PRO- and VEH-treated mice, and ultimate force (Fu), stiffness (S), toughness (U) measured from three-point bending tests also did not differ significantly between groups. Histomorphometric assays also revealed no differences in bone formation or resorption in response to PRO treatment. These data suggest that while developmental perturbation of myostatin signaling through either gene knockout or transgenic inhibition may alter both muscle and bone mass in mice, pharmacological inhibition of myostatin in aged mice has a more pronounced effect on skeletal muscle than on bone.

1. Introduction

Globally, the size of the aging population is increasing rapidly, and as a corollary the prevalence of age-related musculoskeletal disorders such as osteoarthritis, sarcopenia, and osteoporosis is also increasing (Sanchez-Riera et al., 2010). A primary contributor to the morbidity and mortality associated with aging is an increased frequency of falls, and falls are often accompanied by bone fractures. Indeed, falls are the primary factor in more than 90% of bone fractures (Jarvinen et al., 2008). In many cases bone fractures limit subsequent capacity for normal daily activities, ambulation, and independence, ultimately leading to assisted living situations which can be financially burdensome. The disability following a fall and fracture also contributes directly to an increase in comoribities such as respiratory infections, which in turn contribute to greater overall age-related mortality (Bertram et al., 2011).

The growth, development, and aging of muscle and bone are closely linked. Pediatric gains in bone mass are normally preceded by gains in muscle mass, and loss of muscle mass with age typically precedes peak rates of loss in bone density (Hamrick et al., 2010a). The close coupling of muscle and bone across the lifespan therefore suggests that changes in one tissue may be mechanistically linked with changes in another. Indeed, there are multiple mechanisms linking the two tissues such as mechanical loading, muscle-derived trophic factors (myokines), as well as systemic factors such as sex steroids and growth factors that have anabolic effects on both muscle and bone. The functional and perhaps molecular integration between the two tissues therefore suggests that therapeutic strategies targeting one particular tissue may have positive effects on the other, or that certain pharmacologic approaches (e.g., androgen-receptor modulators, vitamin D receptor agonists) could positively impact both tissues at once (Hamrick 2010, 2011, 2012).

Given the very close linkages between muscle and bone referenced above, we sought to test the hypothesis that pharmacologic inhibition of myostatin (GDF-8) could increase both

muscle and bone mass in an aged animal model. Our rationale for pursuing this hypothesis is based on several key observations. The first is that mice lacking myostatin show increased muscle mass as well as increased bone density in the spine, limb, and jaw (Elkasrawy et al., 2010). The second is that recent studies have demonstrated that a myostatin antibody (LeBrasseur et al., 2009; Murphy et al., 2010) and a decoy myostatin receptor (Chiu et al., 2013) can increase muscle mass in aged mice. The decoy receptor (ActR-IIB) was also found to increase bone formation and bone density (Chiu et al., 2013). These findings suggest that therapeutic modulation of myostatin in vivo may be an effective strategy for preserving muscle and bone mass with age, and so we employed a mouse model to evaluate this hypothesis. Specifically, we have previously shown that C57BL6 mice lose significant muscle mass, bone density, and bone strength with age, such that mice 24 months of age show a marked decrease in these measures compared to mice at 12 months of age (Hamrick et al., 2006a). This study utilizes myostatin propeptide treatment in aged (24 months) C57BL6 mice as an in vivo model for assessing the potential effects of myostatin inhibition on age associated muscle and bone loss. The propeptide fragment is utilized here because it has previously been shown to enhance muscle and bone repair in vivo, and binds the active myostatin ligand with high affinity (Bogdanovich et al., 2005; Hamrick et al, 2010b).

2. Materials & Methods

2.1 Production and validation of myostatin propeptide

The myostatin propeptide-Fc fusion protein was produced in CHO cells as described previously (Jiang et al., 2004). To measure the activity of myostatin and the efficacy of the myostatin propeptide a luciferase reporter gene assay was developed where the vector pGL3(CAGA)12 – neo was stably transfected into A204 (human rhabdomyosarcoma) cells. Addition of myostatin to the A204 CAGA cells, and the binding of myostatin to its receptors, initiates the Smad signal

transduction pathway and activates the luciferase reporter gene. The level of activation is proportional to the luciferase activity and the linear portion of the activity curve is in the ng/ml range (Fig. 1), which is what is expected for a protein in the TGFβ superfamily. The addition of the myostatin propeptide prevents the binding of myostatin to its receptors, and the IC50 for the propeptide is approximately 2.0 nM (Fig. 1).

2.2 Animals and treatment for aging study

C57BL6 mice were purchased from the aged rodent colony at the National Institute on Aging, National Institutes of Health (USA) at 22 months of age and delivered to Georgia Regents University, Augusta GA. Animals were allowed to acclimate for one week and were maintained at the Laboratory Animal Service Facility of Georgia Regents University. An earlier dose-response study was used to evaluate the efficacy of a myostatin propeptide in vivo (Hamrick et al., 2010b). Adult mice (5-6 mo.) were treated with the propeptide at 0, 10, 20, or 50 mg/kg at day 0, 5, and 10 and then sacrificed one week after the last treatment. Those data showed that propeptide treatment increased fore- and hindlimb muscle mass by 10% at the 10 mg/kg dose and increased muscle mass by more than 15% at the 20 mg/kg dose, but the 50 mg/kg dose did not increase muscle mass beyond the increase observed in the 20 mg/kg group (Hamrick et al., 2010b). The 20 mg/kg dose was therefore used in this study. Mice were divided into two treatment groups: a vehicle group (VEH; n=14) and a myostatin propeptide group (PRO; n=15). Mice received i.p. injections every five days for 25 days with a dosage of 20 mg/kg body weight at a volume of 0.2 ml. Myostatin propeptide [4.48mg/ml] was obtained from Pfizer Inc (Cambridge, MA, USA). Mice were given calcein i.p. injections to label actively mineralizing bone surfaces four days and 24 hours prior to sacrifice.

2.3 Tissue collection

Animals were euthanized by CO_2 overdose and thoracotomy following procedures approved by the Institutional Animal Care and Use Committee of Georgia Regents University. The extensor digitorum longus (EDL) and soleus (SOL) muscles from one limb were dissected out, cut in half and embedded in OCT for cryostat sectioning and muscle fiber size measurements. The tibialis anterior from one limb was dissected out, weighed, snap frozen and homogenized for RT-PCR of the following myogenic markers: myostatin, Mafbx, Murf 1, MHC, and IGF-1. The right tibias were disarticulated and fixed in 70% ethanol for μCT and plastic sectioning while the left tibias were stored damp at minus 20^{o}C for biomechanical testing followed by RT-PCR for bone formation and osteoblast differentiation markers Runx2, Osx and BMP-2. The femora were fixed in 4% paraformaldehyde, decalcified and embedded in paraffin for sectioning and stained for osteoclasts (TRAP kit from Sigma 386A-1KT) and osteoblasts (Celestine blue/ van Geison's).

2.4 Bone Histomorphometry

Osteoblast and osteoclast counts were performed as previously described (Wenger et al., 2010) on 4–5 μm sections after the specimens were decalcified in 4% EDTA for 1 week, dehydrated, cleared in xylene, then embedded in paraffin. Osteoblasts were counted on sections stained using von Giessen, and osteoclasts counted on sections stained for tartrate-resistant acid phosphatase (TRAP) activity. Standardized peripheral locations from the metaphysis were measured in a fixed region of interest. Mineralizing surfaces were measured from calcein-labeled, undecalcified bone sections. Tibias fixed in 70% ethanol were dehydrated and embedded in methyl methacrylate and sectioned in the horizontal (transverse) plane. Sections were viewed using a Zeiss Axioplan2 fluorescent microscope and captured using a SPOT® digital camera to image labeled bone surfaces. Forming surface was calculated as the percentage of non-eroded, single-labeled surface / total surface × 100 (MS/BS).

2.5 Biomechanical testing and micro-computed tomography (uCT)

Left tibias were stored damp at -20^{o}C before being allowed to thaw at room temperature in PBS for 1 hr. Specimens were tested in three-point bending using a Vitrodyne V1000 Material Testing system as described previously (Hamrick et al., 2006b, 2008). Tibias were mounted antero-posteriorly on stainless steel fixtures 5mm apart, approximately 2.5 mm either side of center. Testing was linear displacement control with a displacement speed of 10 μm/sec using a Transducer Techniques 5 kg load cell. Structural, or extrinsic, properties including ultimate force (Fu; height of curve) and stiffness (S; slope of curve) were calculated from load-displacement curves. MicroCT images of the right tibia were scanned using Bruker Skyscan1174 compact micro-CT (Belgium), software version 1.5 (build 9) using NRecon version 1.6.4.8 for reconstructed images.

2.6 PCR and Western blotting

Total RNA was extracted using Trizol and cDNA was synthesized using Quantitect reverse transcription kit (catalog no. 205310; Qiagen). Expression was analyzed quantitatively by means of the Quantitect SYBR Green PCR kit (catalog no. 204143, Qiagen), and QuantiTect Primer Assays. We used specific primers provided by QuantiTect Primer Assays for Myostatin, Murf1, MaFbx, MHC, IGF-1, Runx2, Osx, BMP-2 and 18S, GAPDH and β-actin (internal controls; Table 1). Half of each extensor digitorum longus muscle was snap-frozen in liquid nitrogen for Western blotting. Each muscle was placed in phosphate buffered saline (PBS) and subjected to homogenization using Fisherbrand Tissuemiser® rotary homogenizer until large pieces of muscle were no longer visible. Samples were subjected to two freeze–thaw cycles to disrupt the plasma membrane then centrifuged briefly. Protein concentrations were measured using a commercial BCA reagent (Pierce, Rockford, IL) to ensure equal loading. 30 μg of proteins from whole tissue lysates were mixed 1:1 with 2× sample buffer and then applied to 4-20 %

polyacrylamide gels. Samples were electrophoretically separated and transferred to nitrocellulose membrane (Invitrogen, Carlsbad, CA). The membranes were incubated with specific primary antibodies MURF1 (Abcam cat. 77577) or MAFbx (Santa Cruz Biotech cat. 166806) and then incubated with anti-mouse or anti-rabbit peroxidase-conjugated secondary antibodies (Santa Cruz, CA). After the incubation, the membranes were washed three times for 15 min each with 1× TTBS solution and then incubated with 1 ml of chemiluminescence reagent (Invitrogen). The protein bands were visualized using X-ray films (Fisher Scientific, Rochester, NY).

2.7 Statistical analysis

Single-factor ANOVA with treatment group as the factor was used to for pairwise comparisons of morphometric and histomorphometric parameters. For analysis of gene expression data, the control genes of 18S and Actin were averaged to obtain an average control gene for muscle tissue while GAPDH was used as the control gene for bone. Difference in control gene Ct expression between GDF-8 and vehicle was assessed using a two-sample t-test. Delta Ct values for each treatment group were calculated as $\Delta Ct = Ct_{Target\ gene} - Ct_{Control\ gene}$. The difference in ΔCt expression between GDF-8 and vehicle was assessed using a two-sample t-test. The magnitude of the difference between the groups was estimated using deltadelta Ct values for each target gene and these were calculated as $\Delta\Delta Ct = \Delta Ct_{GDF\text{-}8} - \Delta Ct_{vehicle}$ and fold change was calculated as 2 to the power $-\Delta\Delta Ct$. SAS® version 9.3 (SAS Institute, Inc., Cary, NC) was used for all analyses and alpha=0.05 was used to determine statistical significance.

3. Results

3.1 Myostatin propeptide increases muscle mass and fiber size in aged mice

Body weight of the vehicle- and propeptide-treated animals was similar at the end of the study (Fig 2A). Each treatment group did, however, lose some weight over the treatment period but this was less dramatic for the treated animals, such that their decrease in body weight from day 0 to day 25 was significantly less than that of the vehicle-treated mice (Fig. 2B). Muscle mass of the tibialis anterior was significantly increased in the treated mice, both absolutely (Fig. 3A) and relative to body weight (Fig. 3B). Fiber size of the predominantly fast-twitch extensor digitorum longus (EDL) muscle was also significantly increased by more than 15% in the treated mice (Fig. 3C,D), whereas the increase in muscle fiber size in the predominantly slow-twitch soleus (SOL) muscle was also increased significantly (Fig. 3E) but by a lesser magnitude (~5%). Propeptide treatment produced a slight but non-significant increase in the expression of myostatin itself, as well as expression of myosin heavy chain and IGF-1 (Fig. 4A). Surprisingly, expression of the ubiquitin ligases Murf1 and Mafbx was significantly increased with propeptide treatment (Fig. 4A), and the PCR data were further validated by Western blot (Fig. 4B).

3.2 Myostatin inhibitor does not alter bone formation or bone strength in aged mice

MicroCT data from the tibia show that bone mineral density is actually slightly higher (3%) in the tibias of vehicle-treated mice (Table 2), but other parameters such as bone volume relative to total volume, trabecular number, and trabecular thickness are similar between the two groups (Table 2). Likewise, three-point bending tests of tibias show that ultimate force, stiffness, and toughness (energy to fracture) are also similar between the vehicle- and propeptide-treated mice (Table 2). Bone histomorphometry data reveal that osteoblast and osteoclast numbers do not differ between the experimental groups (Table 3). Fluorochrome labeling showed double-labels in only three mice from each group, and so single-labeled surfaces were compared.

Actively mineralizing surfaces were also similar between the two groups of mice (Table 3). Gene expression data show no significant differences in the expression of osteogenic genes Osx or Runx2 with propeptide treatment, however the expression of BMP-2 is increased in animals receiving the propeptide (Fig. 4C).

4. Discussion

Pharmacological inhibition of myostatin has, to date, been pursued using a variety of in vivo approaches. These include utilization of myostatin-specific antibodies (Bogdanovich et al., 2002; Wagner et al., 2008; LeBrasseur et al., 2009; Murphy et al., 2010), a decoy myostatin receptor (ActRIIB-Fc; Lee et al., 2005; Bialek et al. 2008; Borgstein et al., 2009; Chiu et al., 2013), and myostatin propeptide (Bogdanovich et al., 2005; Hamrick et al., 2010b). Published data now exist in which each of these therapies has been evaluated in aged rodents, so that some comparison of the different approaches can be undertaken. Our data using a myostatin propeptide are consistent with data from studies using myostatin antibodies, where these myostatin inhibitors were found to have significant, positive effects on muscle mass, fiber size, and muscle force production. Specifically, LeBrasseur et al. (2009) used a slightly higher dose (25 mg/kg) than we used in our study (20 mg/kg), but also used weekly injections of a myostatin inhibitor (PF-354 antibody) over a period of 4 weeks in mice 24 months of age. They too found a moderate (<10%) in muscle mass and a significant increase in muscle mass relative to body weight (+12-17%), as we did for the tibialis anterior muscle relative to body weight (+~15%). Murphy et al. (2010), like LeBrasseur et al. (2009), used weekly doses of the PF-354 antibody but used a lower dose (10 mg/kg) for a longer treatment period—14 weeks of treatment starting in mice aged 18 months. These authors found increases in overall muscle mass (<10%) in the gastrocnemius and quadriceps of the aged mice following 14 weeks of treatment, and a significant increase in (+12%) in muscle fiber cross-sectional area of the tibialis anterior muscle, that were similar in magnitude to the changes we observed with propeptide treatment.

Together, these studies using myostatin antibodies and our study using the myostatin propeptide show similar increases in muscle fiber size and muscle mass using either a low dose (10 mg/kg) over a longer (14 week) treatment period, or a higher dose (20-25 mg/kg) over a shorter treatment period (4 weeks).

Data from in vivo studies using either the myostatin antibody or the propeptide differ in two important ways from those utilizing the decoy myostatin receptor (ActRIIB-Fc). First, a 10 mg/kg dose of ActRIIB-Fc administered twice weekly for four weeks increased tibialis anterior mass by 30% and quadriceps mass by 25% (Chiu et al., 2013). These increases in muscle mass are much greater than those observed with either the myostatin antibody or propeptide, which as noted above generated increases in total muscle mass of <10%. It is possible that these differences could be due to the more frequent administration of the ActRIIB-Fc, but the ActRIIB-Fc dose is much lower than that used in either our study or the study by LeBrasseur et al. (2009), suggesting that the ActRIIB-Fc is a more potent molecule for increasing muscle mass in aged mice. The reason for the greater potency of the ActRIIB-Fc for increasing muscle mass is likely because this molecule can bind several ligands in addition to myostatin, including activin, BMP-3, BMP-7, BMP-9, BMP-10, and GDF-11 (Souza et al., 2008). Some of these molecules, such as activin, are also likely to play a role in regulating muscle mass, which is further indicated by the fact that ActRIIB-Fc treatment can increase muscle mass in mice that lack myostatin altogether (Lee et al., 2005). The second way in which our data differ from those using ActRIIB-Fc is related to the effects on bone. ActRIIB-Fc treatment was previously documented to increase bone formation and bone mass in young, growing mice (Bialek et al., 2008; Yan et al., 2008), and the data from Chiu et al. (2013) are consistent with this earlier report in showing that ActRIIB-Fc increases bone density and serum markers of bone formation in aged mice after just 30 days of treatment. In contrast, our data revealed no bone effects with myostatin propeptide treatment. These data may indicate that, as proposed by Chiu et al.

(2013), the anabolic effects of ActRIIB-Fc on bone are due to antagonizing effects on ligands other than myostatin, such as various BMPs or activin.

Previous work in our lab showed that myostatin can inhibit the proliferation of aged bone marrow stromal cells (Bowser et al., 2013), that bone marrow stromal cells from mice lacking myostatin show increased proliferation (Elkasrawy et al., 2011), and that myostatin can inhibit chondrogenesis in vivo and in vitro (Elksrawy et al., 2012). These data may at least in part explain the increased fracture callus size following osteotomy in mice lacking myostatin (Kellum et al., 2009), and increased fracture callus bone volume in mice treated with myostatin propeptide following osteotomy (Hamrick et al., 2010). That is, myostatin seems to play a key role in musculoskeletal injury repair, one in which myostatin secretion from muscle is elevated following muscle damage, and then mediates the repair response in adjacent bone by modulating progenitor cell proliferation (Elkawrawy et al., 2012). On the other hand, myostatin appears to have a more limited role in mature, intact bone. This is indicated by the fact that myostatin itself is not expressed at a significant level by osteoblasts (Digirolamo et al., 2011), and that myostatin inhibition via propeptide treatment in adult mice does not significantly alter osteoblast number, mineralizing surfaces, or bone strength (Tables 1 and 2). Thus, therapeutic targeting of myostatin specifically via antibody or propeptide treatment may have clinical application in the context of improving muscle mass alone, or improving the healing of muscle and bone following injury, but is not likely to have a significant impact on bone formation in the intact, aged animal. In contrast, the decoy myostatin receptor (ActRIIB-Fc) appears capable of increasing muscle mass, bone formation, and bone strength in aged rodents, suggesting that this molecule may have potential clinical use for age-associated loss of both muscle and bone in the form of sarcopenia and osteoporosis.

Muscle and bone are closely associated spatially and in terms of structure and function during growth, development, and aging. Muscle in particular has been considered a driving

force for bone modeling and remodeling, in that muscle is the primary source of mechanical stimuli for bone and bone tissue is thought to adapt its gross structure in response to muscle-derived stimuli. Thus, targeting muscle therapeutically is thought to be one approach for improving bone health, simply by enhancing the mechanical relationship between muscle and bone. On the other hand, a large portion of osteoporotic fractures do not occur in individuals with low bone density as measured by two-dimensional densitometry, and so fall prevention alone may be another strategy for reducing falls and fall-associated morbidity and mortality in the elderly (Jarvinen et al., 2008). Behavioral interventions such as resistance exercise or nutritional interventions such as vitamin D supplementation (Girgis et al., 2013) may improve muscle strength and/or neuromuscular control and proprioception, perhaps reducing fall risk. The extent to which myostatin inhibition may augment such strategies remains relatively unexplored. Mice are relatively small in body weight and their bones are capable of withstanding loads many times their own body mass--for example it takes more than 2 kg of force to fracture the tibia of a 32 g mouse (Fig. 1, Table 2). Thus, increases in muscle mass in these small mammals may not significantly alter the mechanical environment of their bones. Additional studies in patient populations are needed to determine the extent to which therapeutic targeting of muscle alone via a myostatin antibody or propeptide, perhaps in conjunction with an exercise regimen, could reduce the incidence of bone fractures versus a molecule such as ActRIIB-Fc, that may potentially increase the mass and strength of both muscle and bone.

5. Conclusions

We tested the hypothesis that in vivo inhibition of myostatin using an injectable myostatin propeptide (GDF8 propeptide-Fc) would increase both muscle mass and bone density in aged (24 mo) mice. Our goal was to evaluate this potential therapeutic for its capacity to increase both muscle and bone mass in the setting of age-associated sarcopenia and osteoporosis.

Mice were injected weekly (20 mg/kg body weight) with recombinant myostatin propeptide-Fc (PRO) or vehicle (VEH; saline) for four weeks. The data show that PRO treatment significantly increases muscle fiber size and muscle mass, both absolutely and relative to body weight. In contrast bone volume, bone strength, and histomorphometric parameters of bone formation and bone resorption were unchanged with PRO treatment. Our findings are consistent with previous studies utilizing a myostatin antibody in aged mice showing that targeting myostatin increases muscle fiber size and mass; however, our data differ from work utilizing a decoy myostatin receptor (ActRIIB-Fc) to inhibit myostatin function in that ActRIIB-Fc appears particularly effective at increasing bone density and bone formation whereas the propeptide does not. The anabolic effects of ActRIIB-Fc on aged bone are likely due to the ability of this molecule to antagonize other ligands besides myostatin, such as activin or bone morphogenetic proteins. Clinical trials evaluating the potential of these molecules to prevent falls and fractures are needed to determine the optimal approaches for reducing musculoskeletal diseases and complications in the elderly.

Acknowledgements

Funding for this research was provided by the Congressionally Directed Medical Research Programs, Department of the Army (CDMRP093619) and the National Institute on Aging (P01 AG036675).

References

Bertram, M., Norman, R., Kemp, L., Vos, T. 2011. Review of the long-term disability associated with hip fractures. Inj. Prev. 17, 365-70.

Bialek, P., Parkington, J., Warner, L., St. Andre, M., Jian, L., Gavin, D., Wallace, C., Zhang, J., Yan, G., Root, A., Seeherman, H., Yaworsky, P. 2008. Mice treated with a myostatin/GDF-8 decoy receptor, ActRIIB-Fc, exhibit a tremendous increase in bone mass. Bone 42, S46.

Bogdanovich, S., Krag, T., Barton, E.R., Morris, L.D., Whittemore, L.A., Ahima, R.S., Khurana, T. 2002. Functional improvement of dystrophic muscle by myostatin blockade. Nature 420, 418-21.

Bogdanovich, S., Perkins, K., Krag, T., Whittemore, L., Khurana, T. 2005. Myostatin-propeptide mediated amelioration of dystrophic pathophysiology. FASEB J. 19, 543-549.

Borgstein, N., Condon, C., Yang, Y., Wilson, D., Haltom, E., Lachey, J., Seehra, J., Sherman, M. 2009. Preliminary results from single subcutaneous administration of ACE-031, a form of the soluble activin typeII B receptor, in healthy postmenopausal volunteers. Neuromusc. Disorders, 19, 546.

Bowser, M., Herberg, S., Arounleut, P., Shi, X., Fulzele, S., Hill, W.D., Isales, C.M., Hamrick, M.W. 2013. Effects of the activin A-myostatin-follistatin system on aging bone and muscle progenitor cells. Exp. Gerontol. 48, 290-97.

Chiu, C.S., Peekhaus, N., Weber, H., Adamski, S., Murray, E.M., Zhang, H.Z., Zhao, J.Z., Ernst, R., Lineberger, J., Huang, L., Hampton, R., Arnold, B.A., Vitelli, S., Hamuro, L., Wang, W.R., Wei, N., Dillon, G.M., Miao, J., Alves, S.E., Glantschnig, H., Wang, F., Wilkinson, H.A. 2013.

Increased Muscle Force Production and Bone Mineral Density in ActRIIB-Fc-Treated Mature Rodents. J. Gerontol. A Biol. Sci. Med. Sci., Mar 22. [Epub ahead of print]

Digirolamo, D., Singhal, V., Clemens, T., Lee S-J. 2011. Systemic administration of soluble activin receptors produces differential anabolic effects in muscle and bone in mice. J. Bone Miner. Res. suppl. 1167.

Elkasrawy, M.N., Hamrick, M.W. 2010. Myostatin (GDF-8) as a key factor linking muscle mass and bone structure. J. Musculoskelet. Neuronal. Interact. 10, 56-63.

Elkasrawy, M.N., Fulzele, S., Bowser, M., Wenger, K., Hamrick, M.W. 2011. Myostatin (GDF-8) inhibits chondrogenesis and chondrocyte proliferation in vitro by suppressing Sox-9 expression. Growth Factors 29, 253-62.

Elkasrawy, M., Immel, D., Wen, X., Liu, X., Liang, L., Hamrick, M.W. 2012. Immunolocalization of myostatin (GDF-8) following musculoskeletal injury and the effects of exogenous myostatin on muscle and bone healing. J. Histochem. Cytochem. 60, 22-30.

Girgis, C.M., Clifton-Bligh, R., Hamrick, M.W., Holick, M.F., Gunton ,J.E. 2013. The roles of vitamin D in skeletal muscle: form, function and metabolism. Endocrine Reviews 34, 33-83.

Hamrick, M.W., Ding, K.H., Pennington, C., Chao, Y.J., Wu ,Y.D., Howard, B., Immel, D., Borlongan, C., McNeil, P.L., Bollag, W.B., Curl, W.W., Yu, J., Isales, C.M. 2006a. Age-related loss of muscle mass and bone strength in mice is associated with a decline in physical activity and serum leptin. Bone 39, 845-853.

Hamrick, M.W., Samaddar, T., Pennington, C., McCormick, J. 2006b. Increased muscle mass with myostatin deficiency improves gains in bone strength with exercise. J. Bone Miner. Res. 21, 477-483.

Hamrick, M.W., Ding, K.-H., Ponnala, S., Ferrari, S.L., Isales, C.M. 2008. Caloric restriction decreases cortical bone mass but spares trabecular bone in the mouse skeleton: implications for the regulation of bone mass by body weight. J. Bone Miner. Res. 23, 870-879.

Hamrick, M.W. 2010. Invited Perspective: Myostatin (GDF8) as a therapeutic target for the prevention of osteoporotic fractures. IBMS BoneKey. 7, 8-17.

Hamrick, M.W., McNeil, P.L., Patterson, S.L. 2010a. Role of muscle-derived growth factors in bone formation. J. Musculoskelet. Neuronal Interact. 10, 64-70.

Hamrick, M.W., Arounleut, P., Kellum, E., Cain, M., Immel, D., Liang, L. 2010b. Recombinant myostatin (GDF-8) propeptide enhances the repair and regeneration of both muscle and bone in a model of deep penetrant musculoskeletal injury. J. Trauma 69, 579-83.

Hamrick, M.W. 2011. A role for myokines in muscle-bone interactions. Ex. Sports Sci. Revs. 39, 43-47.

Hamrick, M.W. 2012. The skeletal muscle secretome: an emerging player in muscle-bone crosstalk. Nature Bonekey 60, 1-5.

Jarvinen, T., Sievanen, H., Khan, K., Heinonen, A., Kannus, P. 2008. Shifting the focus in fracture prevention from osteoporosis to falls. BMJ 336, 124-126.

Jiang, M.S., Liang, L.F., Wang, S., Ratovitski, T., Holmstrom, J., Barker, C., Stotish, R. 2004. Characterization and identification of the inhibitory domain of GDF-8 propeptide. Biochem Biophys Res Commun. 315, 525-31.

Kellum, E., Starr, H., Arounleut, P., Immel, D., Fulzele, S., Wenger, K., Hamrick, M.W. 2009. Myostatin (GDF-8) deficiency increases fracture callus size, Sox-5 expression, and callus bone volume. Bone 44, 17-23.

LeBrasseur, N.K., Schelhorn, T.M., Bernardo, B.L., Cosgrove, P.G., Loria, P.M., Brown, T.A. 2009. Myostatin inhibition enhances the effects of exercise on performance and metabolic outcomes in aged mice. J. Gerontol. A Biol. Sci .Med. Sci. 64, 940-8.

Lee, S.J., Reed, L.A., Davies, M.V., Girgenrath, S., Goad, M.E., Tomkinson, K.N., Wright, J.F., Barker, C., Ehrmantraut, G., Holmstrom, J., Trowell, B., Gertz, B., Jiang, M.S., Sebald, S.M., Matzuk, M., Li, E., Liang, L.F., Quattlebaum, E., Stotish, R.L., Wolfman, N.L. 2005. Regulation of muscle growth by multiple ligands signaling through activin type II receptors. Proc. Natl. Acad. Sci. USA 102, 18117-22.

Murphy, K., Koopman, R., Naim, T., Leger, B., Trieu, J., Ibebunjo, C., Lynch, G.S. 2010. Antibody-directed myostatin inhibition in 21-mo-old mice reveals novel roles for myostatin signaling in skeletal muscle structure and function. FASEB J. 24, 4433-42.

Sànchez-Riera, L., Wilson, N., Kamalaraj, N., Nolla, J.M., Kok, C., Li, Y., Macara, M., Norman, R., Chen, J.S., Smith, E.U., Sambrook, P.N., Hernández, C.S., Woolf, A., March, L. 2010. Osteoporosis and fragility fractures. Best Pract. Res. Clin. Rheumatol. 24, 793-810.

Souza, T.A., Chen, X., Guo, Y., Sava, P., Zhang, J., Hill, J.J., Yaworsky, P.J., Qiu Y. 2008. Proteomic identification and functional validation of activins and bone morphogenetic protein 11 as candidate novel muscle mass regulators. Mol. Endocrinol. 22, 2689-702.

Wagner, K.R., Fleckenstein, J.L., Amato, A.A., Barohn, R.J., Bushby, K et al. 2008. A phase I/II trial of MYO-029 in adult subjects with muscular dystrophy. Ann. Neurol. 63, 561-71.

Wenger, K., Fulzele, S., Immel, D., Chao, Y., Freeman, D., Elsalanty, M., Powell, B., Hamrick, M.W., Isales, C.M., Yu, J. 2010. Effect of whole body vibration on bone properties in aging mice. Bone 47, 746-55.

Table.1 Nucleotide sequences of mouse primers used for RT-PCR

Gene	Primer	Reference/Accession Number
GAPDH	CAT GGC CTC CAA GGA GTA AGA GAG GGA GAT GCT CAG TGT TGG	M32599
18S	AGT GCG GGT CAT AAG CTT GC GGG CCT CAC TAA AC CAT CCA	V00851
β-actin	GTT TGA GAC CTT CAA CAC CCC GTG GCC ATC TCC TGC TCG AAG TC	Meredith et al 2011*
Mstn	ACT GGA CCT CTC GAT AGA ACA CTC ACT TAG TGC TGT GTG TGT GGA GAT	NM_010834.2
IGF-1	CAG ACA GGA GCC CAG GAA AG AAG TGC CGT ATC CCA GAG GA	NM_184052
MHC	ACA GTC AGA GGT GTG ACTC AGC CG CCG ACT TGC GGA GGA AAG GTG C	NM_001099635
Murf1	GGAGCAGCTGGAAAAGTCCACC AGCTGCTTGGCACTTGAGAGGA	NM_001039048.2
Mafbx	CAGCTTCGTGAGCGACCTC GGCAGTCGAGAAGTCCAGTC	NM_026346
BMP-2	TGT TTG GCC TGA AGC AGA GA TGA GTG CCT GCG GTA CAG AT	NM_007553.2
RUNX-2	GGA AAG GCA CTG ACT GAC CTA ACA AAT TCT AAG CTT GGG AGG A	NM_009820
Osx	ACT ACC CAC CCT TCC CTC AC ACT AGG CAG GCA GTC AGA CG	AY803733

*Meredith ME, Harrison FE, May JM. Differential regulation of the ascorbic acid transporter SVCT2 during development and in response to ascorbic acid depletion. Biochem Biophys Res Commun. 2011 Nov 4;414(4):737-42.

Table 2. microCT and biomechanical testing of the proximal tibia for mice treated with saline (VEH) or myostatin propeptide (PRO; 20 mg/kg). BMD=bone mineral density, BV/TV=bone volume relative to total volume, Tb.Th=trabecular thickness, Tb.N=trabecular number, Fu=ultimate force, U=energy-to-fracture, S=stiffness.

Parameter	**VEH (n=14)**	**PRO (n=15)**	**p value**
BMD	1.43±0.06	1.38±0.05	.01
BV/TV	6.67±2.37	6.14±2.16	.24
Tb. Th	0.11±0.02	0.11±0.01	.47
Tb. N	0.59±0.14	0.54±0.16	.23
Fu (kg)	2.21±.40	2.18±.34	.39
U (kg/um^2)	740.6±417.5	670.3±309	.31
S (g/um)	4.6±2.0	4.7±2.0	.44

Table 3. Bone histomorphometry data for the distal femur of mice treated with saline (VEH) or myostatin propeptide (PRO; 20 mg/kg).N.Ob/BS=osteoblast number per bone surface, MS/BS=mineralizing surface (single-label) relative to bone surface, N.Oc/BS=osteoclast number per bone surface.

Parameter	**VEH (n=15)**	**PRO (n=14)**	**p value**
N.Ob/BS	27.26±17.49	25.09±9.31	.14
MS/BS	0.41±0.17	0.43±0.13	.34
N.Oc/BS	6.33±2.61	6.18±3.82	.38

Figure Captions

Figure 1. Myostatin-induced luciferase activity declines significantly with increasing concentration of myostatin propeptide. The pGL3(CAGA)12 – neo reporter vector contains 12 CAGA boxes previously reported to be TGF-□-responsive elements (Dennler et al. (1998) EMBO J. 17:3091-3100), a neo resistance gene, and the basic luciferase reporter plasmid pGL3 (Promega Corporation).

Figure 2. Body mass (A) and change in body weight (B) for animals treated with either saline (VEH) or myostatin propeptide (PRO) weekly for over four weeks.

Figure 3. Muscle parameters for mice treated with saline (VEH) or myostatin propeptide (PRO) weekly for a period of four weeks. (A) Tibialis anterior mass, (B) tibialis anterior mass relative to body weight, (C) extensor digitorum longus fiber diameter (EDL), (D) alpha-laminin stained cryostat sections of the EDL, (E) soleus muscle fiber diameter.

Figure 4. Real-time PCR data for mice treated with saline (VEH) or myostatin propeptide (PRO) weekly for a period of four weeks showing increased expression of Murf1 and Mafbx in PRO-treated mice (A), Western blot showing similar increases in Murf1 and Mafbx with PRO-treatmend (B), and increased expression of BMP-2 in mice treated with propeptide (C). *P<.05.

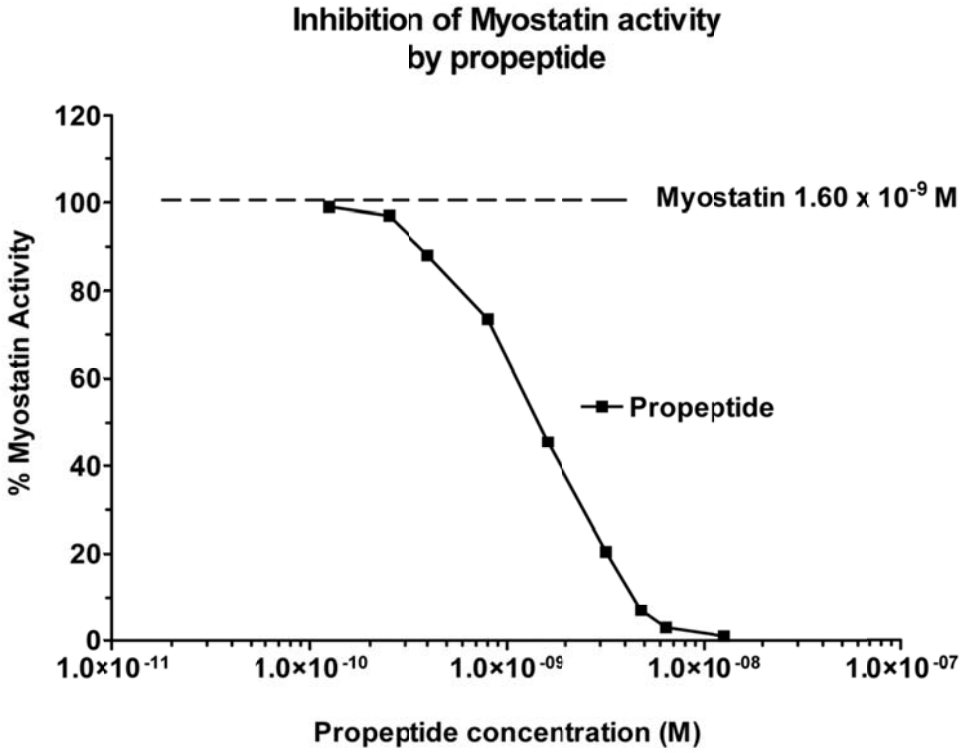
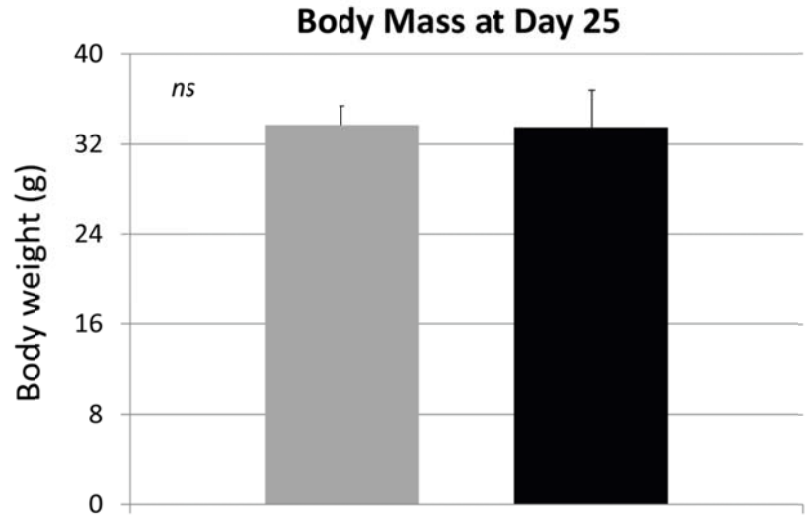
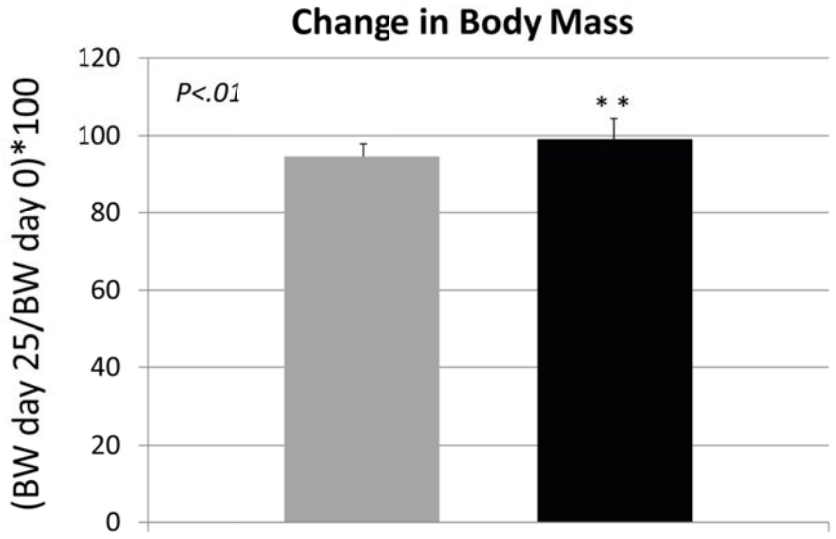

Inhibition of Myostatin activity
by propeptide
120
100
80
60
40
20
0
% Myostatin Activity
Myostatin 1.60 x 10-9 M
Propeptide
1.0×10-11
1.0×10-10
1.0×10-09
1.0×10-08
1.0×10-07
Propeptide concentration (M)

Figure 1

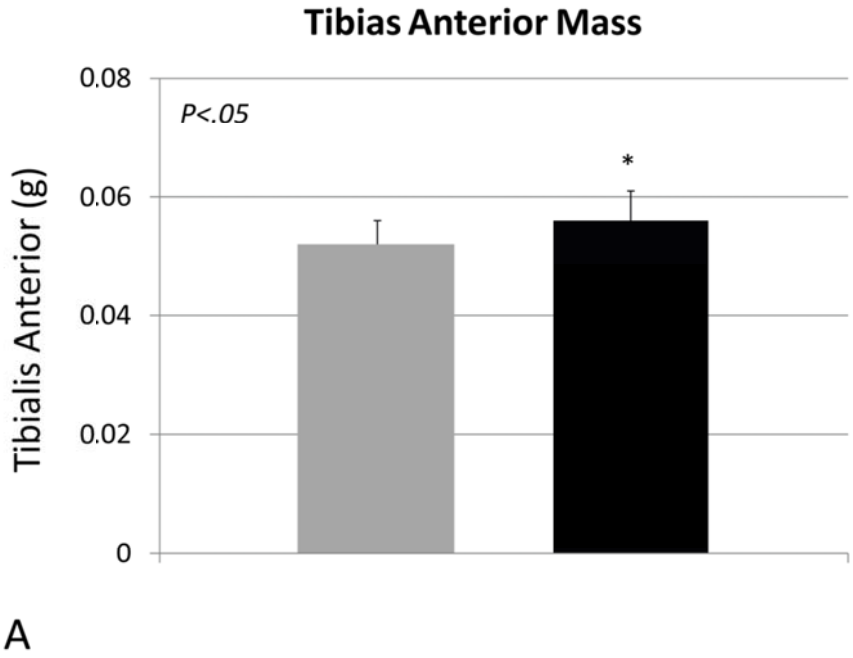

Body Mass at Day 25
40
32
24
16
8
0
Body weight (g)
ns

A

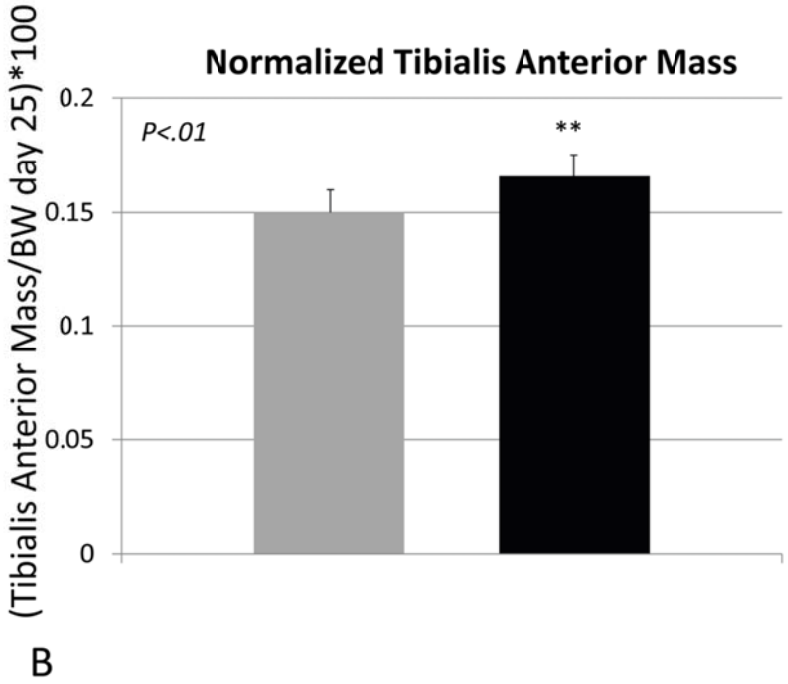

Change in Body Mass
120
100
80
60
40
20
0
(BW day 25/BW day 0)*100
P<.01
* *

B

Figure 2

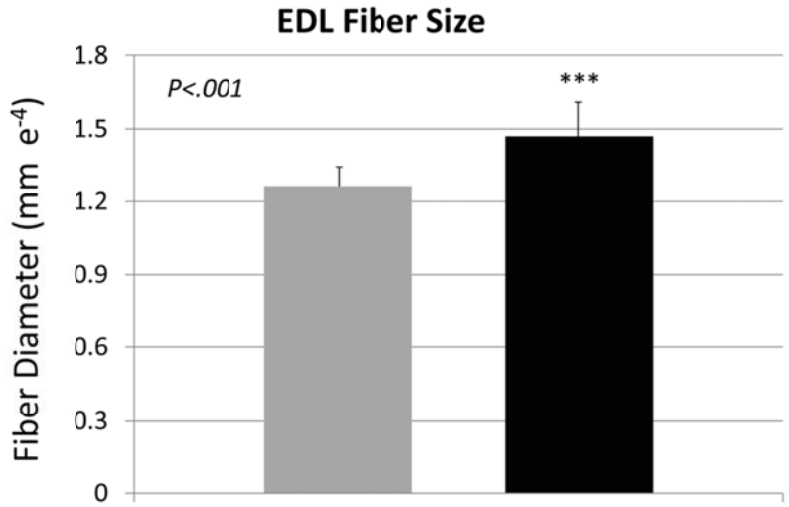


A

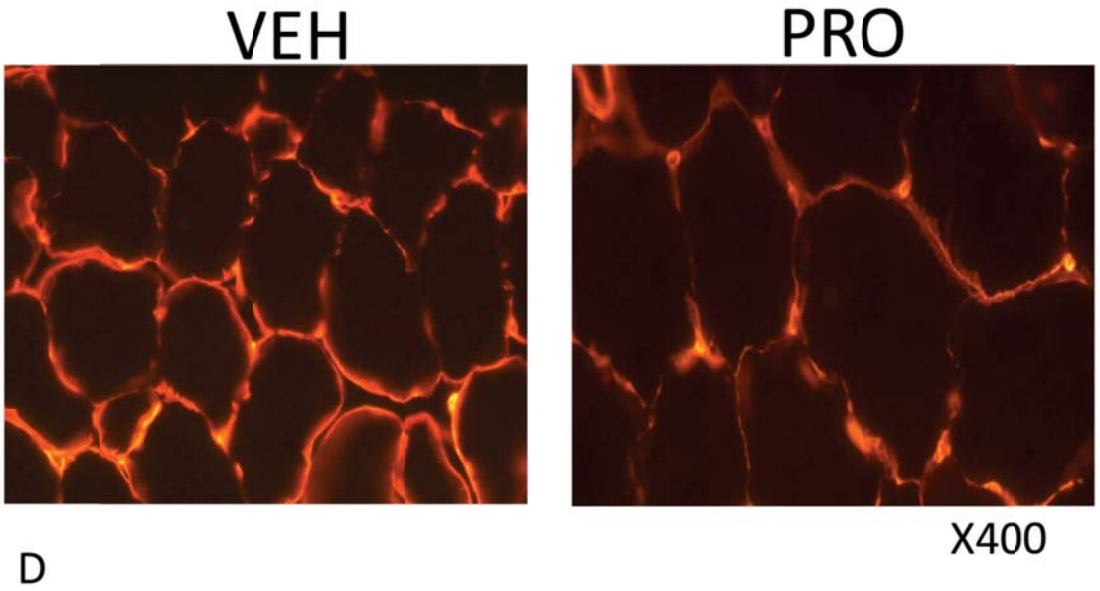


B

Figure 3A,B

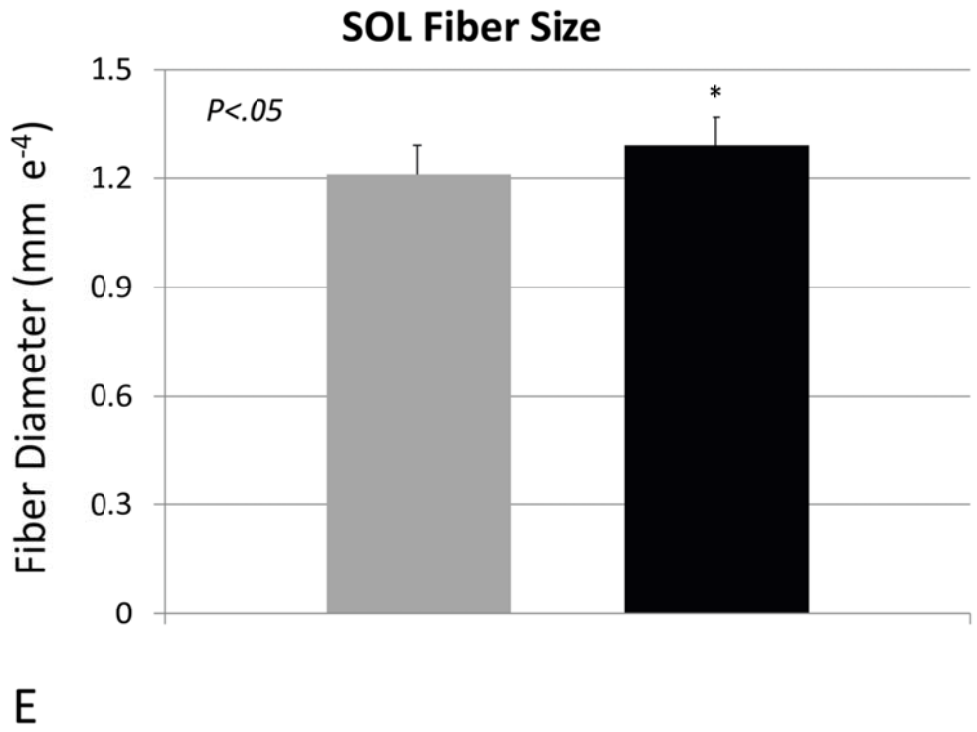


C



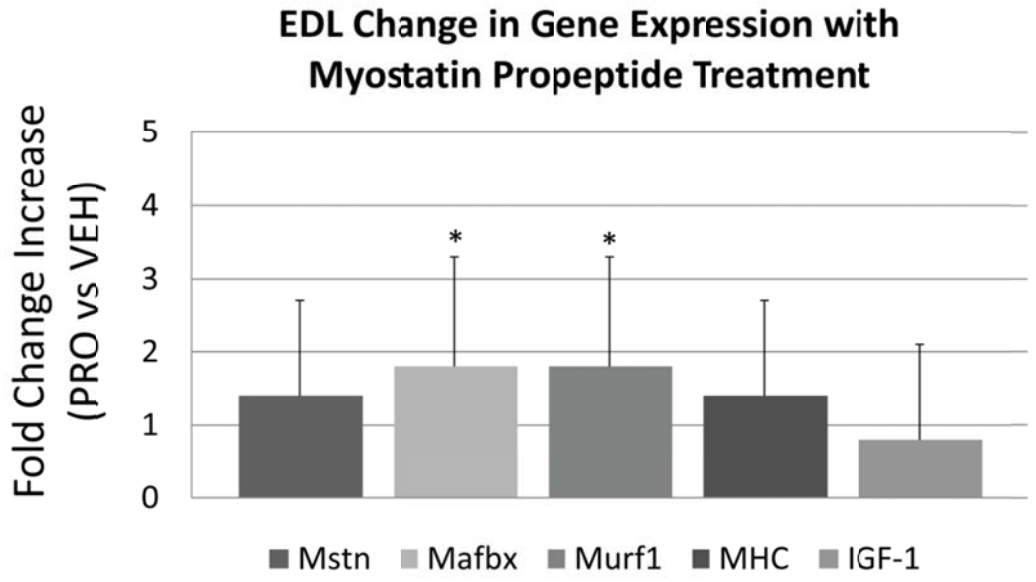
D

Figure 3C,D

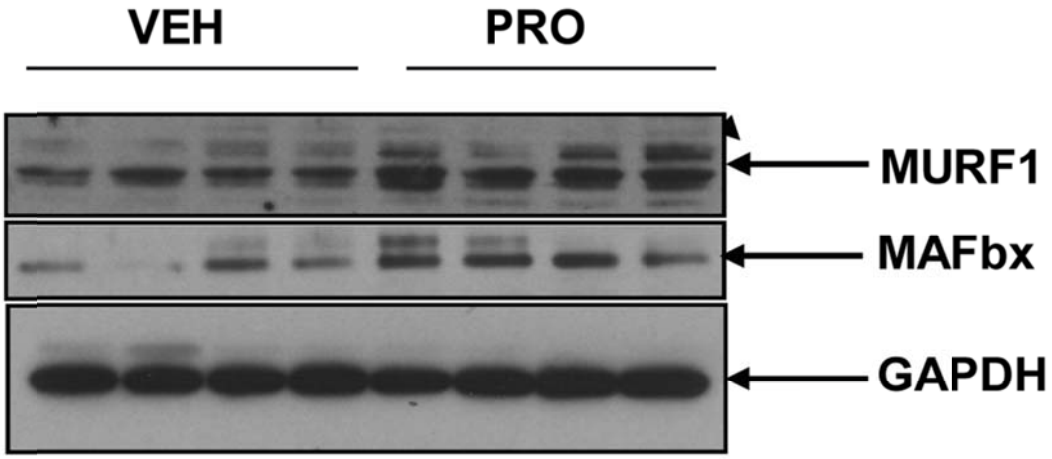


E

Figure 3E

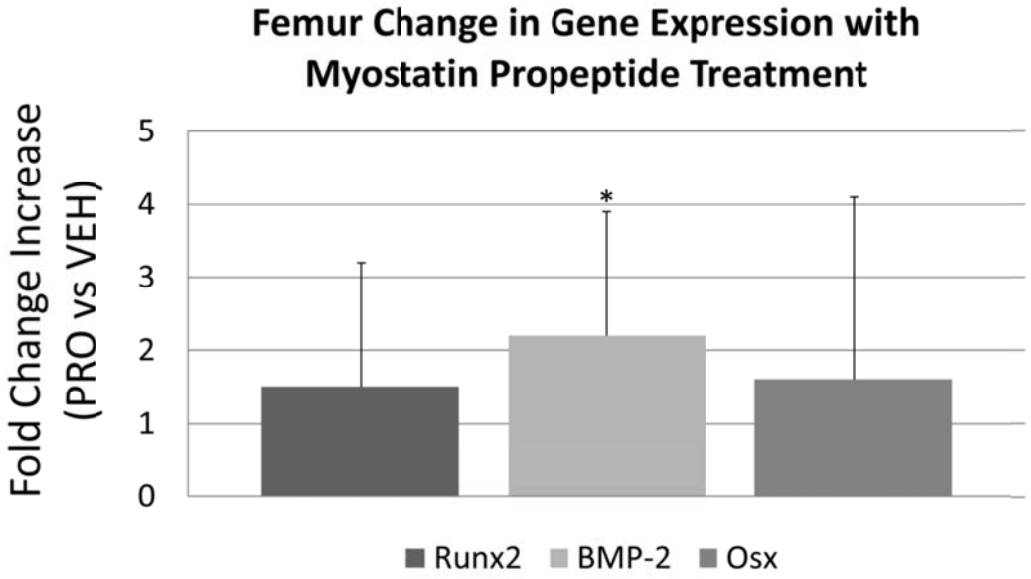


A



B

Figure 4A,B


Femur Change in Gene Expression with Myostatin Propeptide Treatment
Fold Change Increase (PRO vs VEH)
5
4
3
2
1
0
*
Runx2
BMP-2
Osx

C

Figure 4C